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THE ROLE OF NOTCH LIGANDS IN DEVELOPMENT OF THE INNER EAR

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ABSTRACT

The sensory epithelium of the inner ear consists of a mosaic pattern of hair cells separated from one another by supporting cells. The Notch signalling pathway is thought to establish this pattern through a process of lateral inhibition, and has recently been shown to have an early role in inducing sensory patch formation. Several Notch ligands are expressed in the developing sensory patch, but their respective functions in relation to the two roles of Notch signalling are not clear.

This thesis examines the role of two of these Notch ligands, *Delta1* and *Jagged1*, in the development of the inner ear using conditional knockout mice. The effect of loss of these ligands upon hair cell production is strikingly different.

In the absence of *Jagged1*, the total number of hair cells in the cochlea is strongly reduced (although the number of inner hair cells is roughly doubled). This supports the idea that *Jagged1* is required for the early inductive function of Notch in specifying the sensory patches early in development. *Jagged1* conditional knockout mice also exhibit a loss of several of the vestibular sensory patches, with the cristae being most severely affected. Expression of the CDK inhibitor $p27^{Kip1}$ is lost in the mutant cochlea; excess proliferation may thus explain the overproduction of inner hair cells.

In the absence of *Delta1,* auditory hair cells are produced early and in excess, in agreement with the lateral inhibition hypothesis, but, surprisingly, supporting cells are also overproduced. *Delta1* conditional knockout mice also exhibit defects in the vestibular patches. The cristae appear normal, while the maculae are lost or reduced.

These findings confirm that Notch signalling has two distinct functions in the inner ear, for which different ligands are primarily responsible.

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CHAPTER 1

Introduction

The basic components of the classical Notch pathway are a receptor, a ligand, and a transcription factor. Upon binding of a ligand, the intracellular domain of the Notch receptor is released, and enters the nucleus where it acts in a transcriptional complex to modulate gene expression. This simple core pathway lies at the heart of a complicated network of multiple receptors, ligands, and associated regulatory factors, which is required for numerous patterning events during development.

New components of the Notch signalling pathway are constantly being discovered, but some basic questions remain unanswered about those already known. One such question concerns the roles of the multiple Notch ligands found in vertebrates. In this thesis I study the different functions of two Notch ligands, *Delta1* and *Jagged1*, both expressed in the developing sensory epithelium of the inner ear, but in very different patterns in time and space. What parts do these components play in controlling the differentiation of hair cells?

In this introductory chapter, I start by describing the different types of patterning events governed by Notch signalling in other tissues during development. I then review the various components of the Notch signalling pathway in vertebrates, including both those required for Notch activation, and those that modulate production of this signal. I then describe the development of the inner ear, and review what is already known about the role of Notch in this process in zebrafish, chick and mouse. Finally I give a brief review of the other factors known to pattern the developing ear.

1.1 Inhibitory and inductive Notch signalling during development

The Notch signalling pathway enables a cell expressing a Notch ligand to influence gene expression in neighbours that lie in contact with it, expressing the Notch receptor. Notch signalling is required for the patterning of numerous different tissues during development, in almost all animal species studied. The basic Notch signalling mechanism has been adapted to play a variety of roles in different developmental contexts. Notch signalling can act in an inhibitory fashion, preventing cells from adopting the same fate, or in an inductive

fashion, driving cells to adopt the same fate. A differing effect of Notch signalling upon regulation of its ligands lies at the core of inhibitory versus inductive effects of Notch signalling (Figure 1.1). In inhibitory Notch signalling, production of ligand is downregulated in the Notch receiving cell, whereas production of ligand is upregulated in inductive Notch signalling.

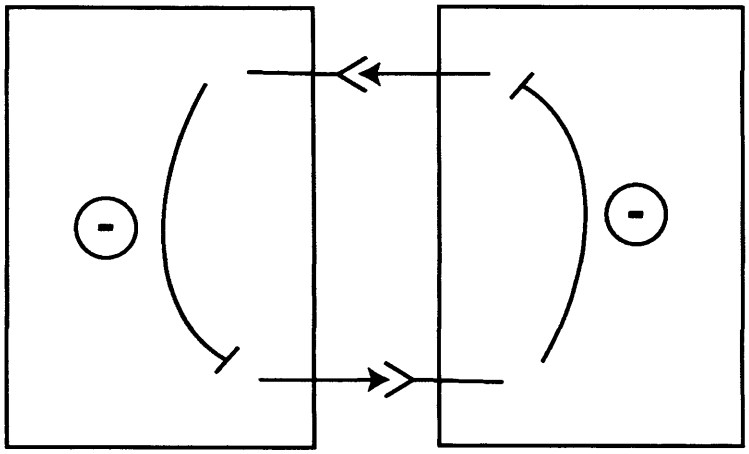
1.1.1 Lateral inhibition

Notch signalling is best known for its role in lateral inhibition, a process first observed in the developing nervous system of grasshoppers. During neurogenesis in the insect central nervous system (CNS), a neuroblast is selected from a pool of ectodermal precursor cells, and then delaminates from the ectoderm. The role of inhibitory cell-to-cell signalling in this process was demonstrated when ablation of a delaminating neuroblast caused a neighbouring cell in the ectoderm to develop as a neuroblast itself (Doe, Kuwada et al. 1985). Since these initial experiments in the grasshopper, insect neurogenesis has been studied in detail in Drosophila, and the central role of Notch signalling in this process has been established through analysis of neurogenic mutants, in which lateral inhibition fails, and neurons are produced in excess.

The mechanism of lateral inhibition in its simplest form is based upon a feedback loop existing between Notch and its ligand, Delta. Activation of Notch by Delta on a neighbouring cell causes the receiving cell to downregulate activity of Delta, and inhibits this receiving cell from becoming neuronal. Therefore, the more Notch activation a cell receives, the less it is capable of delivering, and the less likely it is to become a neuron. In this way, differences in the ability of neighbouring cells to deliver and to receive Notch activation are amplified, with those cells that escape inhibition becoming neurons, and inhibiting neighbouring cells from doing so. In a population of initially equivalent cells, lateral inhibition delivered by Notch signalling mediates competition between neighbouring cells, and causes them to adopt differing developmental fates.

This basic mechanism is subject to many modifications. In Drosophila neurogenesis, for example, it seems that the activation of Delta is not controlled by the amount of Delta mRNA or protein, but by regulation of the expression of other proteins (in particular Neuralized) that are required for Notch function.

(a) lateral inhibition; cells are driven to become different



(b) lateral induction; cells are driven to become similar

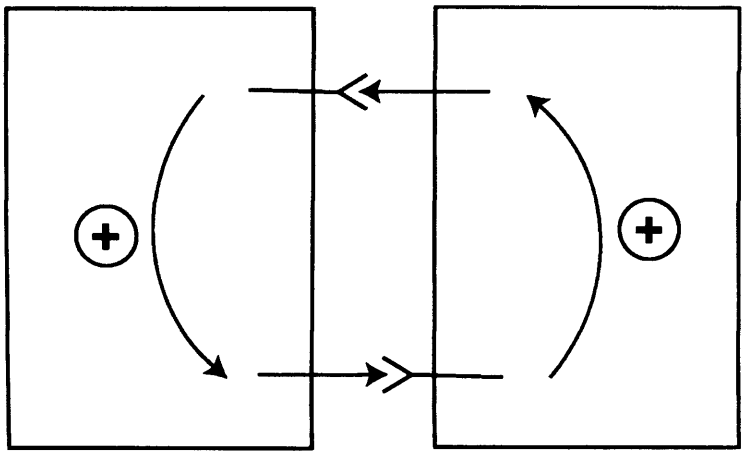


Figure 1.1

Inhibitory and Inductive Notch signalling.

Notch signaling between neighbouring cells can be inhibitory or inductive, either inhibiting or inducing ligand in the Notch receiving cell.
In lateral inhibition (a), a feedback loop is established in which activation of Notch results in an inhibition of ligand, and neighbouring cells are driven to become different. In lateral induction (b), a feedback loop is established in which activation of Notch results in an induction of ligand activity, and neighbouring cells are driven to become similar.

Nevertheless, the role of Notch signalling in neurogenesis is highly conserved, and Delta has been found to mediate lateral inhibition, involving negative regulation of Delta expression by Notch, in the central nervous systems of several vertebrates, including xenopus, chick, zebrafish and mouse (Chitnis, Henrique et al. 1995; Henrique, Adam et al. 1995; Henrique, Hirsinger et al. 1997; Hrabe de Angelis, McIntyre et al. 1997; Haddon, Jiang et al. 1998).

1.1.2 Asymmetric Cell Divisions in Lineage Decisions

In the above version of lateral inhibition, Notch signalling drives initially equivalent cells to adopt different cell fates, generating diversity in a homogeneous population. There are also instances where Notch signalling acts to drive cells to adopt different fates, where the cells response to Notch signalling is already biased. The outcome of the Notch signalling is then predetermined. In other words, Notch signalling is merely the instrument, rather than the generator of diversity.

The most extensively studied case of this is in the mechanosensory bristle lineage of Drosophila, where successive asymmetric division of the sensory organ precursor cell (SOP) gives rise to the various cells of the external sense organ: the socket cell, hair cell, sheath cell and neuron, plus a glial cell. The SOP (or pI) cell divides to give rise to two daughter cells, the pIIa and the pIIb cells. Division of the IIa cell then gives rise to the shaft and socket cells, and division of the IIb cell gives rise to a glial cell, and to the pIIIb cell, which in turn divides to produce the neuron and socket cells (Gho, Bellaiche et al. 1999) Asymmetric cell divisions bias the outcome of Notch signalling between the daughter cells. A loss of Notch signalling during this process, brought about using a temperature sensitive Notch allele, causes all daughter cells to develop as neurons (Hartenstein and Posakony 1990). Both Delta and Serrate ligands are involved in delivering Notch signalling in the sensory organ lineage, apparently acting in a redundant fashion. Loss of Delta alone had only a moderate effect, and loss of Serrate alone did not significantly alter cell fate decisions, whereas loss of both ligands produced a disruption similar to that seen upon loss of Notch (Zeng, Younger-Shepherd et al. 1998).

One of the biasing factors that act to predetermine which cell will escape inhibition by Notch signalling is Numb, a negative regulator of Notch signalling. As the SOP cell divides, Numb protein is asymmetrically distributed to one of the daughter cells, where it blocks Notch activity. The outcome of Notch signalling between the two daughter cells is then biased, so that the cell inheriting Numb escapes Notch activation and develops as the IIb cell. Loss of Numb results in symmetrical division of the SOP, producing two IIa cells, whereas overexpression of Numb causes both daughter cells to become IIb cells (Uemura, Shepherd et al. 1989; Rhyu, Jan et al. 1994). The mechanisms by which Numb inhibits Notch signalling are discussed in more detail later in this chapter.

The role of Numb and asymmetrical cell divisions in the vertebrate CNS is less clear, and remains a controversial area (Roegiers and Jan 2004). However, the two mouse *numb* genes *numb* and *numb-like*, do seem to have a role in neurogenesis. Loss of numb causes embryonic lethality at E11.5, with neural tube defects, and conditional loss of *numb* and *numb-like* results in an overproduction of neurons at the expense of neural progenitor cells (Zhong, Jiang et al. 2000; Petersen, Zou et al. 2002; Petersen, Zou et al. 2004).

1.1.3 Inductive Notch signalling

Notch signalling can also positively regulate the expression of its own ligands, as exemplified in the Drosophila wing. Appendages and other adult external body parts in the fruit fly develop from imaginal discs. The area that gives rise to the wing is divided into dorsal and ventral compartments and the wing margin arises from the boundary between them. Notch activation at this boundary induces key genes required for wing outgrowth and margin formation, including vestigial (vg) and wingless (wg). Notch is activated at the dorsoventral (DV) boundary by both of its ligands, Delta and Serrate. Expression of the selector gene apterous in the dorsal compartment of the wing leads to expression of Serrate in this region, from which it signals to cells in the ventral compartment (Diaz-Benjumea and Cohen 1995; Milan and Cohen 2000). Serrate does not signal via Notch to cells in the dorsal compartment due to the expression of a modulator of Notch signalling, Fringe, within this compartment. Fringe modifies the Notch protein by glycosylation, potentiating activation by Delta, and inhibiting activation by Serrate (Kim, Irvine et al. 1995; Fleming, Gu et al. 1997) (the role of Fringe in Notch signalling is discussed in more detail below). Serrate thus only signals to ventral cells at the DV boundary (de Celis, Garcia-Bellido et al. 1996) . Notch activation in this region upregulates expression of Delta, which

then provokes Notch activation in dorsal cells at the DV boundary (Doherty, Feger et al. 1996) . In contrast to the inhibitory effect of Notch signalling on Delta expression during neurogenesis, Notch signalling positively regulate expression of both of its ligands in the developing wing (de Celis and Bray 1997). This symmetrical activity of Serrate and Delta establishes the zone of Notch activation at the DV boundary.

1.2 The Many Components of the Notch Signalling Pathway

The Notch signalling pathway was originally described in insects and most details of the pathway have been elucidated first in Drosophila or C.elegans, then investigated in vertebrates. Fruit flies have one Notch receptor and two classical Notch ligands: Delta and Serrate. Several other components of the Notch signalling pathway have been identified in screens for genes with neurogenic phenotypes, or with genetic interaction with the Notch pathway (Lehman, Jiménez et al. 1983; Jurgens, Weischaus et al. 1984; Nüsslein-Volhard, Weischaus et al. 1984; Weischaus, Nüsslein -Volhard et al. 1984). Vertebrate homologs of these genes have been found, and their role in Notch signalling investigated. Below, I review the many components of Notch signalling in vertebrates.

1.2.1 The protein structure and post-translational processing of Notch receptors

Mice have four Notch genes; *Notch1-4*. Notch is a transmembrane protein, with numerous EGF-like repeats in its extracellular domain (the number of which varies between homologs) It is through these repeats that Notch interacts with its ligands. After the series of EGF-like repeats, there are three cysteine-rich LN repeats (Notch/Lin-12) external to the transmembrane domain. The LN repeats are thought to prevent activation of Notch before ligand binding. The intracellular domain of Notch (N^{ICD}) is released after activation of the receptor by binding of a ligand, and enters the nucleus, where it modulates target genes. It is composed of a RAM (RBP-Jκ-associated module) domain, followed by six ankyrin repeats, which interact with a key transcription factor described below (Tamura, Taniguchi et al. 1995). The intracellular domain also contains two nuclear-localisation signals, a TAD (transcription transactivation domain, absent in Notch3 and 4) and a PEST (proline, glutamate, serine, threonine) sequence (Figure 1.2).

The Notch receptor is subject to three proteolytic cleavage events at specific sites. The first cleavage, at site1 (S1), occurs before the protein reaches the cell membrane, as it

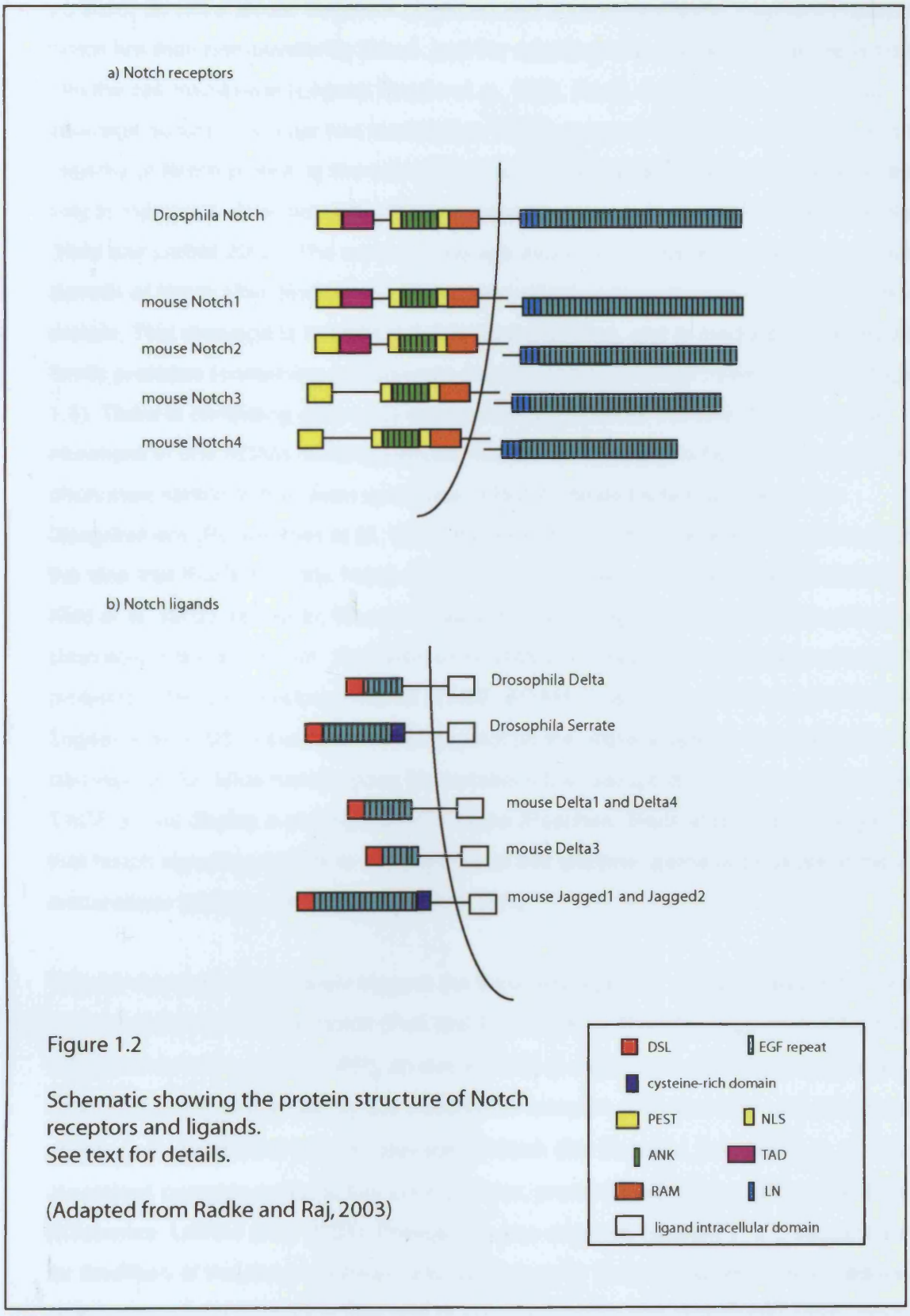


Figure 1.2

Schematic showing the protein structure of Notch receptors and ligands.
See text for details.

(Adapted from Radke and Raj, 2003)

passes through the Golgi network. A furin-like convertase enzyme cleaves Notch into two portions; an extracellular fragment (200kDa) and a transmembrane fragment (120kDa), which are then non-covalently linked, and the resulting heterodimeric molecule is inserted into the cell membrane (Logeat, Bessia et al. 1998, Rand, Grimm et al. 2000). This cleavage occurs in at least two mammalian Notch proteins; Notch1 and Notch2, and the majority of Notch protein at the cell membrane appears to have been processed in this way in mammals. This cleavage does not seem to occur in the case of Drosophila Notch (Kidd and Lieber 2002). The second cleavage event, at S2, occurs in the extracellular domain of Notch after binding of a ligand, and releases the extracellular portion of the protein. This cleavage is thought to follow ligand binding, and is mediated by an ADAM family protease (containing a disintegrin domain and a metalloprotease domain) (Figure 1.3). There is conflicting data as to which enzyme performs this role. In Drosophila, mutations in one ADAM metalloprotease, Kuzbanian (Kuz), give rise to a neurogenic phenotype similar to that seen upon loss of Notch. While there has been some disagreement (Rooke, Pan et al. 1996; Pan and Rubin 1997), recent work has supported the idea that Kuz acts in the Notch receiving cell, mediating S2 cleavage of Notch (Lieber, Kidd et al. 2002). However, the mammalian kuz homolog, ADAM10, is not required for S2 cleavage of Notch (Mumm, Schroeter et al. 2000). Instead, another, closely related, ADAM protease, TNF-α converting enzyme (TACE, ADAM17) appears to be responsible (Brou, Logeat et al. 2000). However, TACE may not be the enzyme wholly responsible for cleavage at S2. Mice homozygous for mutations that disrupt the metalloprotease activity of TACE do not display a neurogenic phenotype (Peschon, Slack et al. 1998), suggesting that Notch signalling occurs in the absence of this enzyme, perhaps because of the other extracellular proteases that can substitute for it.

The S2 cleavage immediately triggers the third cleavage, S3, which is required to release the intracellular portion of Notch (Pan and Rubin 1997). This cleavage resembles that of β-amyloid precursor protein (APP), an event which is defective in Alzheimer's disease, and is normally mediated in part by the γ-secretase complex. This protease complex has been shown to be involved in the S3 cleavage of Notch (De Strooper, Annaert et al. 1999). The γ -secretase complex contains four core proteins; presenilin, nicastrin, APH-1 and PEN-2 (Kimberley, LaVoie et al. 2003). Prescenillin was originally isolated in a C.elegans screen for modifiers of the Notch pathway, and subsequently found to be required for release of Notch intracellular domain in flies and mammals (Levitan and Greenwald 1995; Struhl and Greenwald 1999; Zhang, Nadeau et al. 2000). Thus, mice lacking both mammalian

Figure 1.3

Schematic showing the main Notch signalling pathway components.
(Adapted from Baron, 2003.)

After binding to a ligand, the Notch receptor undergoes two cleavage events. ADAM/TACE cleaves Notch in the extracellular domain, releasing the extracellular fragment of Notch. This cleavage facilitates cleavage of Notch by the gamma-sectretase complex, composed of four core proteins (presenillin, nicastrin, APH-1 and PEN-2).

The intracellular domain of Notch, NICD, is then released from the membrane, and moves to the nucleus. Here, it causes disassembly of a co-repressor complex, containing NICD itself, the transcription factor CSL, SMRT (silencing mediator of retinoid and thyroid hormone receptors) and a histone deacetylase (HDAC-1). The presence of NICD in the nucleus promotes formation of a co-activator complex, again containing NICD, CSL, with histone acetylases and Mastermind. This complex activates transcription genes of the Hes/Hey families.

The intracellular domain of Delta is also important in Notch signalling. Interaction of this domain of the ligand with Neuralised/Mindbomb promotes activation of Notch.

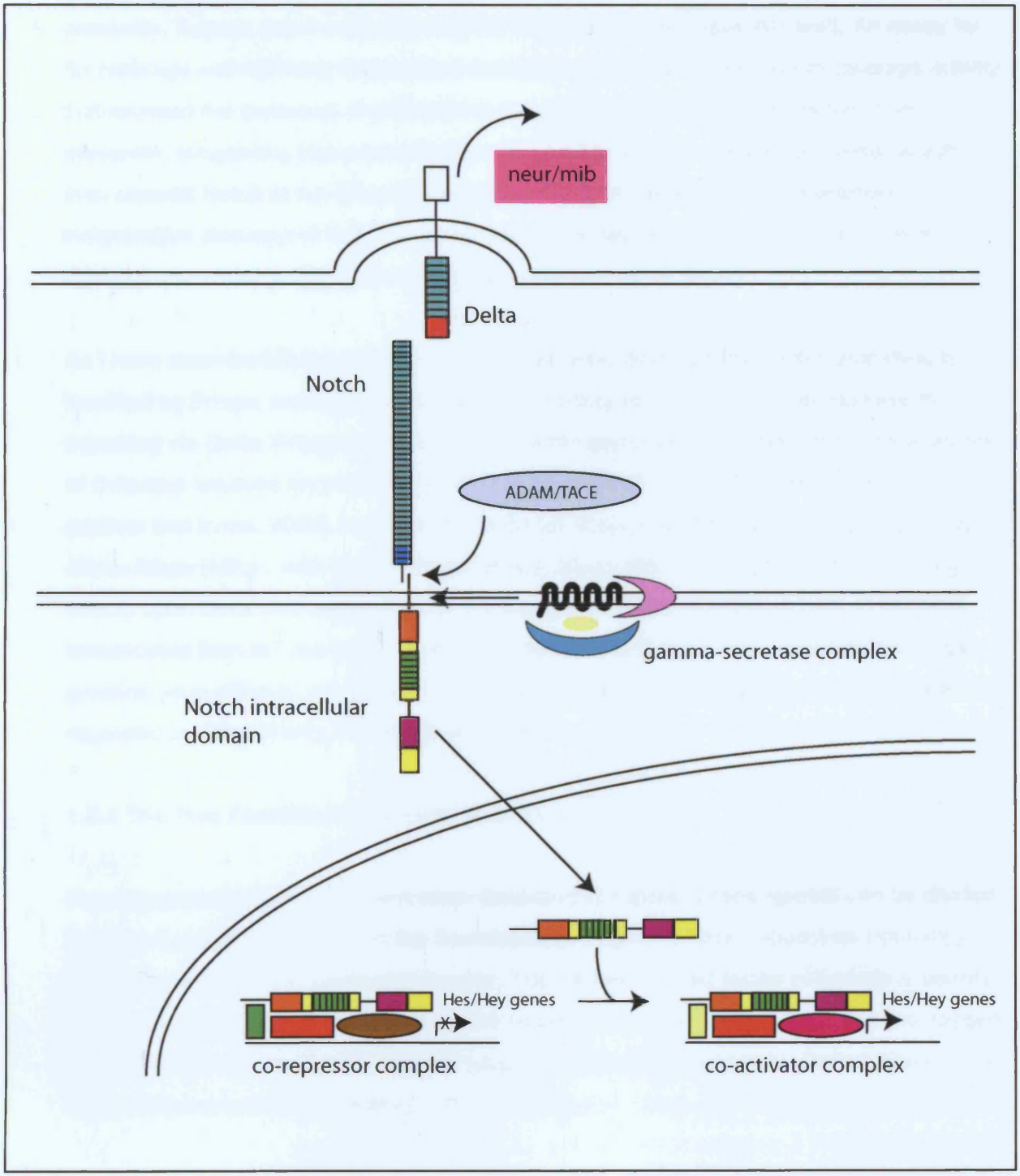


Figure 1.3
Schematic showing the main Notch signalling pathway components.
(Adapted from Baron, 2003)

presenilin homologs, PS-1 and PS-2, die at E9.5 with defects reflecting loss of Notch function (Donoviel, Hadjantonakis et al. 1999). While the S3 cleavage appears to require presenilin, there is some evidence that it is not mediated by presenilin itself. An assay for S3 cleavage activity using fractionated membrane lysates revealed Notch cleavage activity that required the presence of presenilin in the cells, but was physically distinct from presenilin, suggesting that presenilins are required to activate another protease, which then cleaves Notch at the S3 site (Taniguchi, Karlstrom et al. 2002). A presenilin-independent cleavage of Notch has also been reported, which appears to occur at a different site, distal to the S3 site (Berechid, Kitzmann et al. 2002).

As I have described in the context of Drosophila wing development, Notch signalling is modified by Fringe, causing an inhibition of signalling via Serrate, and an increase in signalling via Delta. Fringe is an *O*-fucose specific glycosyltransferase, which adds GlcNAc to *O*-fucose residues on particular EGF-repeats of Notch, modifying ligand interactions (Haines and Irvine. 2003). In mammals there are three fringe genes, *Lunatic fringe* (*Lfng*), *Manic fringe* (*Mfng*), and *Radical fringe* (*Rfng*). These different fringe genes have different effects upon Delta and Jagged ligand interactions with Notch, which appear to be more complicated than in Drosophila. A recent paper reports that the three mammalian fringe proteins have different effects upon Delta1 and Jagged1, with both ligands being positively regulated by Rfng (Yang, Nichols et al. 2004).

1.2.2 The Two Families of Classical Notch Ligands

Five classical Notch ligands have been described in mouse. These ligands can be divided into two families: the Delta and the Serrate/Jagged ligands, based upon their homology to the Drosophila ligands, Delta and Serrate. The Serrate/Jagged family of ligands is usually referred to as Jagged in mouse, so will be referred to as such here. Mice have two Jagged ligands, *Jagged1* (*Jag1*) and *Jagged2* (*Jag2*), and three Delta ligands, *Delta1*, *Delta3* and *Delta4* (also known as *Delta-like1*, *Dll1*, etc.).

The protein structure of Delta and Jagged ligands is very similar. Both are single-pass transmembrane ligands that have a large extracellular domain, and a smaller intracellular domain. The extracellular domain of both Delta and Jagged ligands contain the highly conserved DSL (Delta, Serrate, Lag) domain, followed by a stretch of EGF-like repeats (Figure 1.2). The DSL domain has been found to be the minimal unit required for

interaction of these ligands with Notch receptors. For a stable interaction, the DSL domain is required in conjunction with the first two EGF-like repeats (Shimizu, Chiba et al. 1999) The two families of ligands differ in that the members of the Jagged family contain a greater number of EGF-like repeats, and a cysteine-rich domain in close proximity to the cell membrane (Fleming 1998). The functional significance of this difference is not clear.

The intracellular domains of the Delta family show no obvious sequence similarity to those of the Jagged family, but there is evidence that this part of both types of Notch ligands are functionally important. Both ligands appear to be proteolytically processed in a similar way to the Notch receptor, by an ADAM protease and the γ -secretase complex (LaVoie and Selkoe 2003). The functional significance of this processing of Notch ligands has not been extensively studied, but it appears that the released intracellular domains of the two families of ligands are able to regulate gene expression, at least in cell culture systems. LaVoie et al also reported that the Jagged1 intracellular domain (J^{ICD}), can antagonise N^{ICD} activity. The intracellular portion of Delta, and possibly of Jagged, is important in an additional way, as a target for ubiquitination (see below).

The Notch signalling pathway has been adapted to play numerous different roles during developmental patterning. One level of specialisation is the presence of multiple Notch receptors and ligands, which vary in their binding affinities for one another, in their patterns of expression and in the way they are regulated. Experiments in vitro suggest that ligands can activate multiple receptors, albeit with different strengths of interaction. For example, Jagged1 is capable of binding and activating (in the order of binding affinity) Notch3, Notch2 and Notch1 (Shimizu, Chiba et al. 1999). Several Notch receptors and ligands have been found in the same tissues during development with differing patterns of expression. A study of the expression of Notch ligands and receptors in the CNS found receptor-ligand pairs that are expressed in overlapping domains. For example, in the eye *Jagged1* and *Notch3* are both expressed in the ciliary margins of the retina, whereas *Delta1* and *Notch1* are expressed in the central region of the retina (Lindsell, Boulter et al. 1996). However, the combination of receptors and ligands is not always so straightforward. As I shall discuss below, there are several cases, the inner ear being one of them, where the patterns of expression suggest that different ligands have differing functions in the patterning of tissues.

1.2.3 Alternative Notch pathway activation

Although in most circumstances Delta and Jagged seem to function as transmembrane cell surface molecules, soluble forms of Delta and Jagged Notch ligands have also been found to interact with Notch both in vitro and in vivo. The effects of these interactions vary, with activation or inhibition of Notch signalling seen in different situations (Qi, Rand et al. 1999; Small, Kovalenko et al. 2001). Recently, a secreted form of Jagged1 that lacks the transmembrane and intracellular domains has been found expressed in keratinocytes, where it has been reported to promote their differentiation (Aho 2004). If this is correct, it may represent a novel mechanism for long-range Notch activation during development.

Members of the F3/Contactin family of glycosyl phosphatinositol (GPI)-anchored neural cell adhesion molecules have been shown to be capable of acting as Notch ligands (Hu, Ang et al. 2003; Cui, Hu et al. 2004). Both F3/Contactin itself, and another family member, NB-3, promote development of oligodendrocytes through activation of Notch. These atypical ligands do not simply mimic the activity of classical Notch ligands. NB-3 and F3/Contactin bind to a different regions of the extracellular domain of Notch, and have differing effects upon developing oligodendrocytes. Whereas Notch activation mediated by Jagged1 inhibits differentiation of oligodendrocyte precursor cells (OPCs), activation mediated by NB-3 and F3/Contactin is required for differentiation and maturation of oligodendrocytes (Hu, Ang et al. 2003; Cui, Hu et al. 2004).

1.2.4 Regulation of gene expression by N^{ICD}

Once released from the membrane, the N^{ICD} can enter the nucleus. Its presence here alters the activity of a CSL transcription factor (CBF-1 (or RBP-Jκ) in mammals, Su(H) in Drosophila and LAG1 in C.Elegans). In the absence of N^{ICD}, CSL interacts with corepressors, including SMRT (silencing mediator of retinoid and thyroid hormone receptors) and a histone deacetylase (HDAC-1) (Kao, Ordentlich et al. 1998). The transcriptional complex formed in the nucleus with N^{ICD} is not yet fully understood, though several factors involved have been identified. As previously stated, the presence of N^{ICD} in the nucleus appears to prevent interaction of the CSL transcription factor with co-repressors and instead forms with it an activator complex, containing histone acetylases (p300 and PCAF), and Mastermind (Wallberg, Pedersen et al. 2002).

Although CSL is thought to mediate the action of N^{ICD} in the majority of cases, there is some evidence for activation of Notch target genes that does not require the CSL transcription factor (Martinez Arias, Zecchini et al. 2002). In Drosophila, the phenotypes observed upon loss of Notch are more severe than those seen upon loss of Su(H) (Rusconi and Corbin 1999). There also appears to be a CSL-independent role of Notch in verebrates. For example, it has been reported that differentiation of mammalian myogenic cells is blocked by Notch signalling, even in the presence of a dominant negative form of CSL (Nofziger, Miyamoto et al. 1999).

Mastermind is a nuclear protein, thought to be required for N^{ICD} mediated gene activation, and identified originally as the product of a neurogenic gene in Drosophila (Lehman, Jiménez et al.1983). There are three Mastermind homologs in mouse and humans, called MAML1-3 (Wu, Kobayashi et al. 2004). MAML proteins form a complex with N^{ICD} and CBF-1 (Jeffries, Robbins et al. 2002), in which MAML binds to ankyrin repeats in the intracellular fragment of Notch via its N-terminal domain. The three MAML proteins appear to interact with intracellular domains from the different Notch receptors with differing affinity, and therefore have differing effects upon the regulation of Notch target genes (Wu, Aster et al. 2000). Thus, differential expression of mastermind genes would produce different levels of gene activation by N^{ICD}.

The different Notch proteins themselves may have differing effects upon transcription. The intracellular domain of Notch3 binds to the transcription factor CBF-1, but was found to be a weaker transcriptional activator than Notch1 (Beatus, Lundkvist et al. 1999). There is some evidence that the intracellular domain of Notch3 may compete with that of other Notch proteins for the CBF-1 transcription factor, and for co-activators required for Notch-mediated gene activation (Beatus, Lundkvist et al. 1999), and in this way might act as a repressor of canonical Notch signalling. This possibility is supported by the finding that overexpression of Notch3 had the same effect upon development of the pancreas (inducing premature differentiation of endocrine cells) as does loss of Delta1 or of CBF-1 (Apelqvist, Li et al. 1999).

1.2.6 Direct targets of Notch signalling

The downstream targets of Notch signalling include the Hes (Hairy / Enhancer of split (E(spl) homologs) and Hey (Hairy/E(spl)-related) (also known as HRT (Hairy-related

transcription factor) and HERP (HES-related repressor protein)) families of basic helix-loop-helix (bHLH) transcription factors. Seven *Hes* genes and three *Hey* genes have been described in mammals (reviewed in Iso, Kedes et al. 2003). Both Hes and Hey family members act to repress transcription, with the exception of Hes6, which antagonises Hes1 activity (Bae, Bessho et al. 2000). Their targets include pro-neural genes such as *Math1*. They thus mediate the inhibition of neuron production seen upon Notch activation.

The *Hes* genes are thought to repress transcription in two ways: directly and indirectly. Direct repression is mediated by homodimers of Hes proteins, which bind to the DNA and recruit corepressors. In Drosophila these include Groucho, which in turn recruits a histone deacetylase, which may mediate transcriptional repression by alteration of the chromatin structure (Paroush, Finley et al. 1994). A similar system may operate in mammals via the mammalian Groucho homolog, TLE (transducin-like E(spl)) (Grbavec and Stifani 1996). Alternatively, Hes family members can repress transcription indirectly by sequestering proteins required by other bHLH transcription factors in transcriptional activation.

Hey proteins do not repress transcription via Groucho, but recruit the large mSin3 complex, including N-CoR and HDAC1 (Iso, Sartorelli et al. 2001). They are also capable of repressing transcription indirectly, again by interactions with proteins required for transcriptional activation. Hes and Hey family members have been shown to form heterodimers, which are more potent repressors of transcription than Hes or Hey homodimers (Iso, Sartorelli et al. 2001).

1.2.7 Regulation of Notch signalling by endocytosis and targeted degradation

Notch signalling has been found to be regulated at various levels by the endocytosis of ligands and receptors, and by the targeted degradation of members of the Notch signalling pathway through the activity of E3 ubiquitin ligases. Polyubiquitination of a protein, that is, ligation of numerous ubiquitin molecules to the protein, targets it for degradation. Monoubiquitination can be a signal for other fates, including endocytosis. Addition of ubiquitin occurs in three stages, mediated by three types of enzymes: E1 – E3. Firstly, ubiquitin-activating enzymes (E1) act upon the ubiquitin molecule. The activated ubiquitin is then transferred to a ubiquitin-congugating enzyme (E2). Finally, an E3 ubiquitin ligase interacts with both the E2 enzyme and the target protein to attach the ubiquitin molecule (Hershko and Ciechanover 1998).

Activation of Notch can be regulated by factors affecting endocytosis of the Notch proteins themselves, and of their ligands. *Neuralised* (*Neur*) was originally identified in Drosophila as a neurogenic gene required for Notch signalling, and has since been shown to be an E3 ubiquitin ligase that ubiquitylates Delta and thereby promotes endocytosis and degradation of Delta (Parks, Klueg et al. 2000; Deblandre, Lai et al. 2001; Lai, Deblandre et al. 2001; Yeh, Dermer et al. 2001). There is dispute as to whether Neur is required in the cell receiving the Notch signal, or in the cell delivering the Notch signal (Pavlopoulos, Pitsouli et al. 2001; Seugnet, Simpson et al. 1997)). In the former case, a possible interpretation would be that Neur-dependent degradation of Delta prevents an inhibitory interaction of Notch with Delta *in cis.* According to the opposite view, where Neur is required in the cell delivering the Notch-activating signal, the suggestion is that ubiquitylation by Neur somehow activates Delta as a Notch ligand. The latter view is in better accord with the observed expression pattern of neuralised.

The situation is clearer for *mindbomb* (*mib*), a gene isolated in the Tübingen and Boston mutagenesis screens. Disruption of *mib* results in a severe neurogenic phenotype, indicating a requirement in Notch signalling (Jiang, Brand et al. 1996; Schier, Neuhauss et al. 1996). Mib has since been identified as an E3 ubiquitin ligase which, just like Neuralised, interacts with the intracellular domain of Delta, causing Delta to be endocytosed and degraded, and has been shown to be necessary to enable Delta to activate Notch in neighbouring cells (Itoh, Kim et al. 2003).

In the Drosophila bristle lineage, Neuralised protein is asymmetrically inherited at certain cell divisions, and biases the outcome of lateral inhibition, in such a way that the cell inheriting Neur adopts the "neural-like" fate (Le Borgne and Schweisguth 2003). As mentioned earlier, numb is another asymmetrically localised, cell fate determinant that acts through an effect on the Notch pathway, but by action on Notch itself: Numb is a cell-autonomous inhibitor of Notch activity. The mechanism by which Numb inhibits Notch activity is not yet fully understood. It may act in part by promoting endocytosis of Notch, reducing the amount of Notch at the cell surface. Evidence for this comes from the finding that numb interacts with α-adaptin, part of a complex that causes endocytosis of transmembrane receptors (Berdnik, Torok et al. 2002). It may also – or alternatively - inhibit Notch activity by targeting Notch for degradation through recruitment of the E3 ubiquitin ligase, Itch (McGill and McGlade 2003). Numb is itself regulated by another E3

ubiquitin ligase, LNX, which thus acts as a positive regulator of Notch signalling, removing the inhibition caused by Numb (Nie, McGill et al. 2002).

Yet another E3 ubiquitin ligase that acts as a regulator of Notch signalling is Supressor of deltex (Su(dx)) (Qiu, Joazeriro et al. 2000; Fostier, Evans et al. 1998). This protein acts to negatively regulate Notch signalling by interacting with and promoting ubiquitination of Notch. Su(dx) was so named because it suppresses the phenotype of *deltex* (*dx*), which encodes a cytoplasmic protein identified in a Drosophila screen for genetic interaction with the Notch pathway (Xu and Artavanis-Tsakonas 1990; Busseau, Diederich et al. 1994). Experiments in Drosophila suggested that Deltex positively regulates Notch by binding to the ankyrin-repeats in the N^{ICD} (Matsuno, Diederich et al. 1995). Recent experiments indicate that Deltex acts to positively regulate Notch signalling not at the cell membrane, but in the late endosomal compartment (Hori, Fostier et al. 2004).

Though Notch can be detected at the cell membrane, detection of the N^{ICD} in the nucleus has proved to be more difficult. This appears to be due to the rapid degradation of the N^{ICD} through the activity of another E3 ubiquitin ligase, Sel-10. Sel-10 was isolated in a C.elegans screen for negative regulators of Notch signalling, and was found to interact with the C-terminus of Notch. The mammalian homolog has also been isolated, and has been found to target the intracellular portion of Notch1 for degradation. Treatment of cells with molecular blockers of the proteosome and with a dominant negative form of Sel-10 stabilises N^{ICD}, and increases transcription of Notch target genes (Gupta-Rossi, Le Bail et al. 2001; Oberg, Li et al. 2001; Wu, Lyapina et al. 2001). Sel-10 thus appears to provide an "off switch" that tightly regulates N^{ICD} activity in the nucleus.

1.3 *Jagged1* and *Delta1* are co-expressed in several different tissues during development

This study focuses on two different Notch ligands in mouse: Jagged1 and Delta1. Both ligands are necessary for embryonic development beyond mid-embryonic stages, with both *Jagged1* and *Delta1* knockout mice dying at around E10.5 with vascular defects (Hrabe de Angelis, McIntyre et al. 1997; Xue, Gao et al. 1999). This early embryonic lethality is also seen in mice homozygous for mutations of Notch1, or of the CSL protein CBF-1 (Oka, Nakano et al. 1995; Huppert, Le et al. 2000). Aside from the apparently similar effects on the vasculature, the defects in development caused by loss of the two

Notch ligands are very different, consistent with their differing patterns of expression during development.

Expression of these two ligands is seen in many different tissues during development, often in complementary patterns. They are seen in defined domains that overlap with one another (eg. the otic vesicle), in domains with abutting boundaries (eg. the retina), and in more fine-grained, salt-and-pepper patterns within a tissue (eg. inner ear sensory epithelium). A particularly striking example of the complementary pattern of expression of Delta1 and Jagged1 is seen in the spinal cord. Here the ligands are expressed in separate longitudinal stripes, within which their expression is punctate and transient, and appears to be marking different populations of nascent neurons (Myat, Henrique et al. 1996).

Notch signalling controls keratinocyte proliferation and differentiation in the developing epidermis (Lefort and Dotto 2004). Both *Delta1* and *Jagged1* are expressed in the developing epidermis, along with *Jagged2*, *Notch1* and *Notch2*. The expression of *Delta1* and *Jagged1* within the skin differs, and they appear to play very different roles in the patterning of this tissue. *Delta1* is expressed in the population of keratinocyte stem cells, where it is thought to maintain the stem cells, and signal to Notch expressing cells outside the Delta1-positive clusters of stem cells, causing them to proliferate and differentiate (Lowell, Jones et al. 2000). In contrast, *Jagged1* is coexpressed with *Jagged2*, *Notch1* and *Notch2* in differentiating keratinocytes, where it is thought to promote terminal differentiation. Notch signalling is also required for the control of keratinocyte proliferation (Nickoloff, Qin et al. 2002). Conditional inactivation of *Notch1* in the developing mouse epidermis, results in increased levels of proliferation, a process that is usually inhibited by Notch-mediated induction of the cell cycle inhibitor, $p21^{Cip1}$ (Rangarajan, Talora et al. 2001;Nicolas, Wolfer et al. 2003).

Delta1 and Jagged1 are also found in the developing vasculature. Again, multiple Notch receptors and ligands are expressed in this tissue during development. *Notch1*, *Notch3*, *Notch4*, Delta1, *Delta4*, *Jagged1* and *Jagged2* are all expressed in arteries, but not in veins at E13.5 (Villa, Walker et al. 2001, and unpublished data, this lab). Some of these receptors and ligands are segregated to separate cell types, with Notch3 being restricted to the smooth muscle cells, and Delta4 and Notch4 restricted to the endothelium. *Jagged1*, however, appears to be expressed in both cell types. Notch signalling is thought to be required in vasculature remodelling (modification of the primary vascular plexus into veins,

arteries and capillaries) and in arterial versus venous specification (reviewed in Shawber and Kitjewski 2004). Mice with mutations in Notch1 or Jagged1 exhibit defects in vascular remodelling and die midgestation (Xue, Y., X. Gao, et al. (1999). A role for Notch in arterial/venous specification has been demonstrated in zebrafish, where loss of Notch in an embryonic artery results in loss of arterial markers, whereas activation of Notch inhibits expression of venous markers (Lawson, Scheer et al. 2001).

In this section I give a description of the expression patterns of *Jagged1* and *Delta1* during development, and of the defects in development associated with their loss. A detailed description of the expression of *Jagged1* and *Delta1* in the developing ear is given in chapter 2, and the effect of heterozygous loss of the ligands upon ear development is discussed in the context of other Notch pathway mutants later in this chapter.

1.3.1 The role of *Jagged1* during vertebrate development

The earliest reported expression of *Jagged1* in the mouse embryos is at E7.5, when it is seen in the embryonic endoderm surrounding the primitive streak. A day later, *Jagged1* transcript is detected in the hindbrain in rhombomeres R1, R3 and R5, but by E9.5 expression in the hindbrain has disappeared. A day later, at E10.5, expression is seen in scattered cells of the telencephalon and midbrain and in cells lining the fourth ventricle. Expression of *Jagged1* in scattered cells of the telencephalon and midbrain is seen over the next three days of development. By E15.5, it becomes restricted to the ventricular layer of the neuroepithelium, an area associated with proliferating cells. Expression is also seen in the dorsal root ganglia at E10.5, and in sharply defined, longitudinal stripes along the spinal cord. Low levels of expression can be seen at the border of developing somites. In the rat eye, *Jagged1* is expressed in the lens and in the ciliary margins of the retina at E12.5. By E14.5, expression has become restricted to the equatorial zone and the anterior epithelium of the lens (Lindsell, Boulter et al. 1996). Expression is also seen in the choroid and hyaloid plexus of the eye, presumably localised to the developing blood vessels, as in other tissues.

At E9.5 *Jagged1* is strongly expressed in the first branchial cleft, between the first and second branchial arches. This tissue later contributes to the epithelium lining the external auditory canal and to the tympanic membrane (Kaufman and Bard 1999). By E10.5 it is also expressed in maxillary and mandibular processes of the first branchial arch and in the second branchial arch at E12.5, which contribute to the upper and lower jaw, and to the

middle ear ossicles. *Jagged1* is also expressed in thickenings in the ectoderm called the ectodermal placodes.

A hybridisation signal was detected throughout the heart in wholemount rat embryos at E12.5. By E13.5, it has become restricted to the aorta, the pulmonary trunk and the coronary arteries. In mouse, Mitsiadis et al (1997) report expression of *Jagged1* in the aorta, and in "most other blood vessels" in mouse. This finding is consistent with the homozygous knockout phenotype of *Jagged1*, where defects in vascular development lead to early embryonic lethality (Xue, Gao et al. 1999).

The expression pattern of *Jagged1* described in rodents is almost identical to that seen for *Serrate1*, the chick homolog of *Jagged1* (Myat, Henrique et al. 1996).

1.3.2 Disruption of *Jagged1* results in a range of developmental defects

The *Jagged1* loss of function phenotype has been described in *Jagged1*^{dsl} mice, in which 5kb of sequence from the 5' part of the gene has been removed, including the coding sequence for the DSL domain. The homozygotes have severe defects in vascular development which are thought to cause the embryonic death seen at E10.5 (Xue, Gao et al. 1999). The yolk sacs of homozygous *Jagged1*^{dsl} mice appear pale and lack large blood vessels, indicating a defect in vascular remodelling.

Mutations of *Jagged1* have been found to be the cause of Alagille syndrome in humans, an autosomal dominant disorder affecting 1:70,000 live births (Li, Krantz et al. 1997). This syndrome is characterised by defects of the heart, liver, eye, kidney, face and skeleton. Defects of the heart range in severity from a narrowing of the outflow tract of the heart, to more complex defects affecting the formation of the septa and valves. Interestingly, inner ear defects have also been reported in Alagille patients, with a loss of auditory and vesibular function. CT scans reveal truncations of the anterior and posterior semicircular canals (Le Caignec, Lefevre et al. 2002). This inner ear defect is strikingly similar to that seen in mice heterozygous for a mutation in *Jagged1*, described in section 1.6.

Mice heterozygous for a loss of *Jagged1* do not display all the developmental defects found in people with Alagille syndrome. The syndrome is more closely phenocopied by

mice that are doubly heterozygous for both *Jagged1* and *Notch2,* which display defects in heart, kidney and liver (McCright, Gao et al. 2001; McCright, Lozier et al. 2002).

1.3.3 The role of *Delta1* in development

Delta1, like *Jagged1*, is expressed in several different tissues during development. The earliest reported detection of expression of *Delta1* by in situ hybridisation is at E7.5, when it is seen in the posterior mesoderm and the primitive streak of the embryo (Bettenhausen, Hrabe de Angelis et al. 1995). Expression is maintained in this region, during somite formation, with high level expression in the presomitic mesoderm, and stripes of transient expression in the caudal parts of the developing somites. Like *Jagged1*, but more extensively, *Delta1* is expressed in nascent neurons of, giving a salt-and-pepper pattern of *Delta1* positive and negative cells in the forebrain and midbrain (E8.5), in the hindbrain (E9.5), and in longitudinal stripes along the length of the neural tube (E9.5). These stripes are complementary to those of *Jagged1* in this part of the developing nervous system. Also like *Jagged1*, *Delta1* is expressed in the ectodermal placodes, including the otic and nasal placodes, and the placode giving rise to the cranial ganglia. At E10.5, additional sites of expression are seen in the dorsal root ganglia and the neural crest (Bettenhausen, Hrabe de Angelis et al. 1995).

Delta1 transcripts have been detected in the developing kidney: in the mesonephric mesoderm at E9.5, and in the mesonephric tubules at E10.5. Analysis of the pattern of *Delta1* expression in the kidney of older embryos was done using a $Delta1^{LacZ}$ reporter line in which the β-galactosidase (β-Gal) coding sequence was inserted downstream from the *Delta1* promoter (Hrabe de Angelis, McIntyre et al. 1997; Beckers, Clark et al. 1999). X-Gal staining in $Delta1^{LacZ}$ heterozygous embryos, which appear phenotypically normal, stained cells of the mesonephric mesoderm at E13.5 and E15.5, and was maintained in the kidney during nephrogenesis. Staining was also seen in the developing lung, pancreas and spleen, in the developing epidermis, whisker and hair follicles, and the mesenchymal cells of the palatal rugae.

Expression of *Delta1* was also detected in the developing heart (Bettenhausen, Hrabe de Angelis et al. 1995), and LacZ staining was detected in $Delta1^{LacZ}$ heterozygotes in the endothelium of all blood vessels studied (Beckers, Clark et al. 1999). This pattern of

Delta1 expression is consistent with the haemorrhaging seen in *Delta1* homozygous knockout mice, which results in early embryonic lethality.

Mice homozygous for a knockout mutation of *Delta1* die at E10.5 with defects in the developing vasculature (Hrabe de Angelis, McIntyre et al. 1997). As would be expected based upon the expression pattern of *Delta1*, these mice also exhibit defects of the developing nervous system and somites. The central nervous system in *Delta1* homozygous embryos is hyperplastic, with a huge overproduction of neurons, consistent with the role Delta1 is thought to play in mediating lateral inhibition during neurogenesis. Somitogenesis is also disrupted, in that the borders of somites do not form correctly.

1.4 The development of the inner ear is a feat of complex patterning on the gross structural and on the cellular level

In this study, I have used the mouse inner ear as a model system in which to study the differing roles of *Delta1* and *Jagged1* in tissue patterning. In this section I describe the mature structure of the murine inner ear and give an overview of how this elaborate structure is formed during development, reviewing as I do so the genes thought to pattern the developing ear.

1.4.4 Mature structure and function of the inner ear

The inner ear is a complex 3-D structure that houses the vestibular and auditory sensory epithelia. The sensory epithelia of the inner ear are composed of mechanosensory hair cells, and their surrounding, non-sensory supporting cells. The mature morphology of the inner ear is shown in Figure 1.4.a. The three semi-circular canals, the saccule and the utricle house the vestibular sensory patches. Superior, posterior and lateral semi-circular canals detect angular acceleration in three orthogonal planes. Each canal terminates with a bulbous ampulla that houses the sensory patches, the cristae ampullae. Movement of endolymph within a semicircular canal displaces the gelatinous cupula that overlies the crista, and this movement deflects the hair cell bundles. Two pouches, the saccule and utricule, in the central vestibule of the inner ear contain the maculae, the sensory patches that are responsible for detecting linear acceleration. Vertical and horizontal acceleration are detected by the saccular macula and the utricular macula, respectively. Adjacent to the saccule is the cochlea. This coiled structure houses the auditory sensory epithelium, the

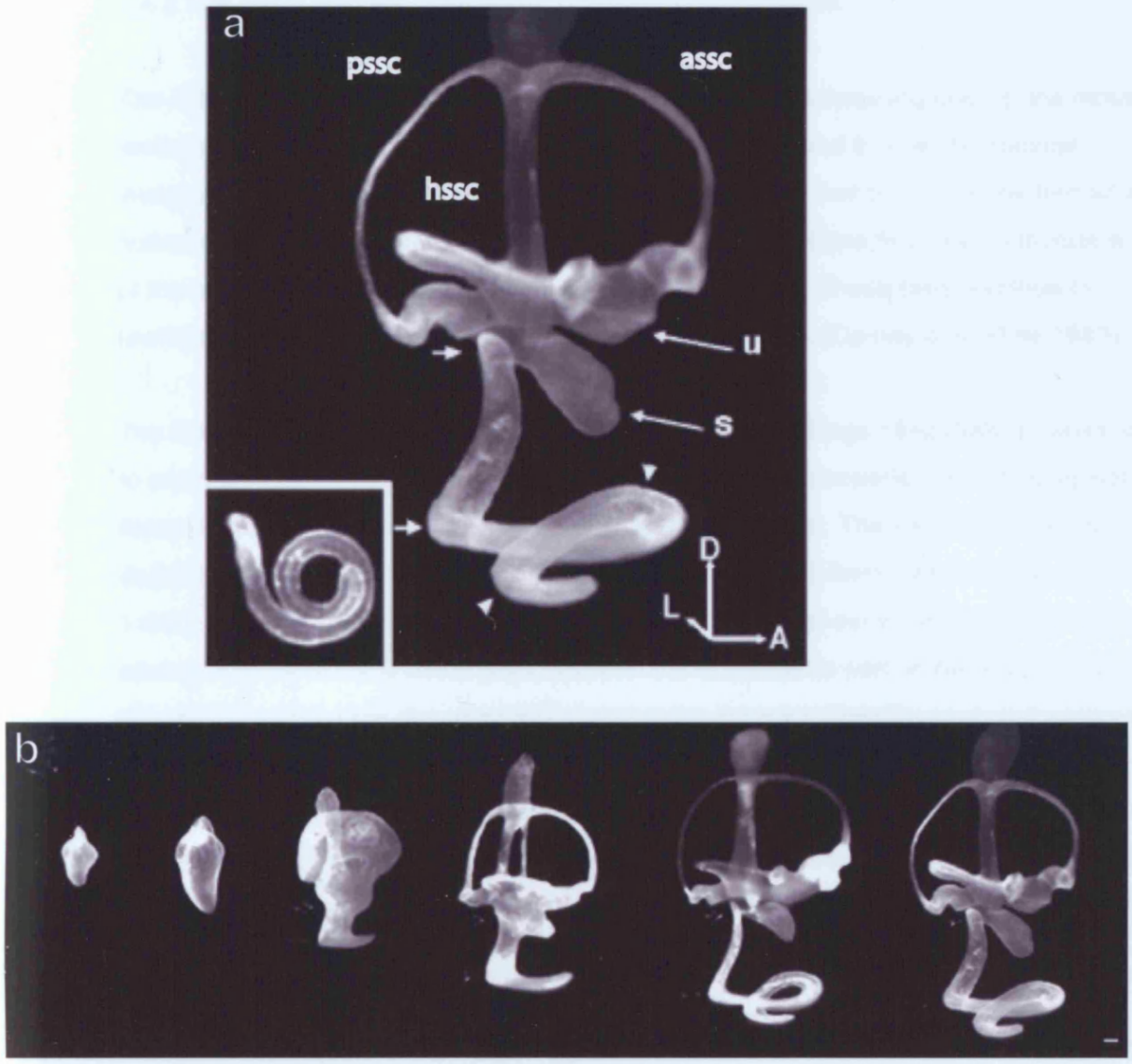


Figure 1.4

The structure of the mouse inner ear and its development

(a) The structure of the mouse inner ear at E17.5 revealed by paintfilling. The vestibular apparatus comprises the posterior (pssc), anterior (assc) and horizontal (hssc) semicircular canals, the utricle (u) and the saccule (s). The coiled cochlea can be observed below, and in boxed image. (b) Paintfills of the inner ear at various embryonic stages, illustrating the gross structural development. Stages are (from left to right) E10.5, E11.5, E12.5, E13.5, E15.5, E17.5. Scale bar in (b) is 100µm.
(From Morsli, Choo et al. 1998)

organ of Corti (Hudspeth 1989). The cochlea undergoes progressive coiling during development, until it reaches its mature one and three quarter turns.

1.4.2 The inner ear develops from an ectodermal placode

The inner ear arises from the otic placode, an ectodermal thickening seen in the mouse embryo at E8.5 parallel to the junction of rhombomeres 5 and 6. The otic placode invaginates and detaches from the overlying ectoderm, so that by E9.5 it has formed a hollow epithelial ball called the otocyst. Neuroblasts delaminate from the ventrolateral wall of the otocyst at E10.5, and stream away from the otocyst. These cells continue to proliferate and contribute to the cochleovestibular ganglion (Carney and Silver 1983).

The three semi-circular canals are produced from outpouchings of epithelium, which begin to protrude from the otocyst from E11.5. As development proceeds, the opposing sides of these hollow plates come into contact in their central region. The two sides fuse and degenerate, leaving only the edges of the discs, which then form patent canals (Figure 1.4.b.). This occurs sequentially. The superior and posterior semi-circular canals form first, arising from the vertical canal plate, found in the dorsolateral part of the otocyst. Fusion of the vertical canal plate occurs at E12, forming the superior, then the posterior semicircular canal, and the central crus commune. The horizontal semicircular forms last, from the horizontal canal plate. By E12, the utricle is identifiable, with the saccule appearing a day later, at E13.5. These structures that house the vestibular sensory patches are all in place by E13, and have achieved their mature morphology by E15 (Martin and Swanson 1993; Morsli, Choo et al. 1998).

The rudiment of the cochlea is first identifiable at around E11.5 as a protrusion from the ventral part of the otocyst. Outgrowth of the cochlear rudiment is accompanied by progressive coiling of the structure, until the full one and three-quarte turns is achieved at E17.5 (Sher 1971; Morsli, Choo et al. 1998). A zone of mitosis has been found in the base of the cochlea (Marovitz 1976), and it has been suggested that outgrowth of the cochlea occurs as this zone increases in size. Those cells produced first in this zone end up in the apex, and those formed later end up in the base.

1.4.3 Origin of the sensory patches

A detailed analysis of the expression of two genes that are thought to be early markers of the vestibular and auditory sensory patches in the mouse otocyst has been performed by (Morsli, Choo et al. 1998) They describe the expression pattern of *Bone Morphogenetic Protein 4 (Bmp4)* a marker of the cristae, and *Lunatic Fringe (Lfng)*, a marker of the maculae. Both genes are also expressed in the developing cochlea.

At E10.25, *Bmp4* expression is seen in two patches, one in the posterior, and the other in the anterolateral part of the otocyst. By E12, the anterior patch of expression has divided into anterior and lateral patches, which correspond to the anterior and horizontal cristae. The posterior patch has also divided, giving a posterior patch corresponding to the future posterior crista, and a patch that corresponds to the developing cochlea. This pattern of early *Bmp4* expression in the inner ear of mouse, restricted to the cristae and cochlea, differs from the pattern reported in the inner ear of chick, where expression of *Bmp4* is seen throughout all the prospective sensory patches (Wu and Oh 1996). As the cochlear rudiment of the mouse elongates and coils, *Bmp4* is expressed along its greater curvature. Expression of *Bmp4* in the cristae persists until E16, when its expression is downregulated in the developing hair cells, but maintained in the supporting cell population. At this stage it is also seen in supporting cells of the two maculae. By postnatal stages, *Bmp4* expression in the cochlea could be seen at the outer margin of the sensory patch, perhaps in Hensen's cells, and in the Claudius cells.

Lfng expression in the early otocyst is restricted to its anteroventral portion, in a domain separate from the anterior *Bmp4* expressing patch. At E11.5, the expression domain of *Lfng* overlaps with that of *Bmp4*, but covers a broader domain in the anterior otocyst. The patch of *Lfng* expression divides at E12, giving rise to dorsal and ventral patches, which seem to mark the macula utriculi and the developing cochlea and macula sacculi, respectively. Complementary to *Bmp4* expression in the greater surface of the developing cochlea, *Lfng* is expressed at its lesser curvature. Their expression overlaps only at the apex of the cochlea rudiment. Later, at E13, their expression also overlaps in the cristae, becoming restricted to the supporting cells of all vestibular patches at E16, as hair cells

develop. In the cochlea, expression of *Lfng* also becomes restricted to the supporting cell population.

It is not clear which, if either, of the expression patterns mark the future sensory patch in the developing cochlea before hair cell differentiation has begun. Based upon the complementarity of the *Bmp4* and *Lfng* expression patterns, Morsli et al suggested that they specify sensory and non-sensory domains of the epithelium, with *Lfng* marking the sensory domain, and *Bmp4* marking a domain lateral to this, with overlap perhaps occurring in the Hensens' cells.

1.4.4 Cell differentiation in the sensory patches of the inner ear

The auditory hair cells of the mammalian cochlea are arranged in four separate rows, which extend along its length. A single row of inner hair cell lies closest to the inner margin of the coil. Lateral to these cells are three rows of outer hair cells. All hair cells are separated from one another by supporting cells, of which there are several different sub-types in the mammalian cochlea (Figure 1.5). Inner hair cells are surrounded and separated from one another by inner phalangeal cells. External to the row of inner hair cells is a row of distinctive supporting cells called the inner and outer Pillar cells. These cells have rectangular apical surfaces that are easily identifiable on the surface of the sensory epithelium. In the mature organ of Corti, these cells form the lateral walls of the tunnel of Corti. Each outer hair cell has an accompanying Deiters' cell. These supporting cells comprise three rows interspersed between the outer hair cell rows, separating the hair cells from one another. At the lateral edge of the sensory patch lie the Hensens' cells. These are tall, broad cells, whose apical surfaces stand in contrast to the sensory patch cells, and the numerous, small, hexagonal Claudius cells which stretch away to the lateral wall of the cochlea duct.

Cells that will form the auditory sensory patch exit the cell cycle sequentially – apical first, basal last - between E11.5 and E15.5, with the majority of cells undergoing their terminal mitosis between E13.5 and E15.5 (Ruben 1967). Hair cells and supporting cells are thought to have a common progenitor. Evidence for this comes from studies in the developing chick basilar papilla (the equivalent of the mammalian organ of Corti), where progenitor cells of the developing sensory patches could be labelled using a replication-defective retrovirus expressing a histochemically detectable marker. When cells were

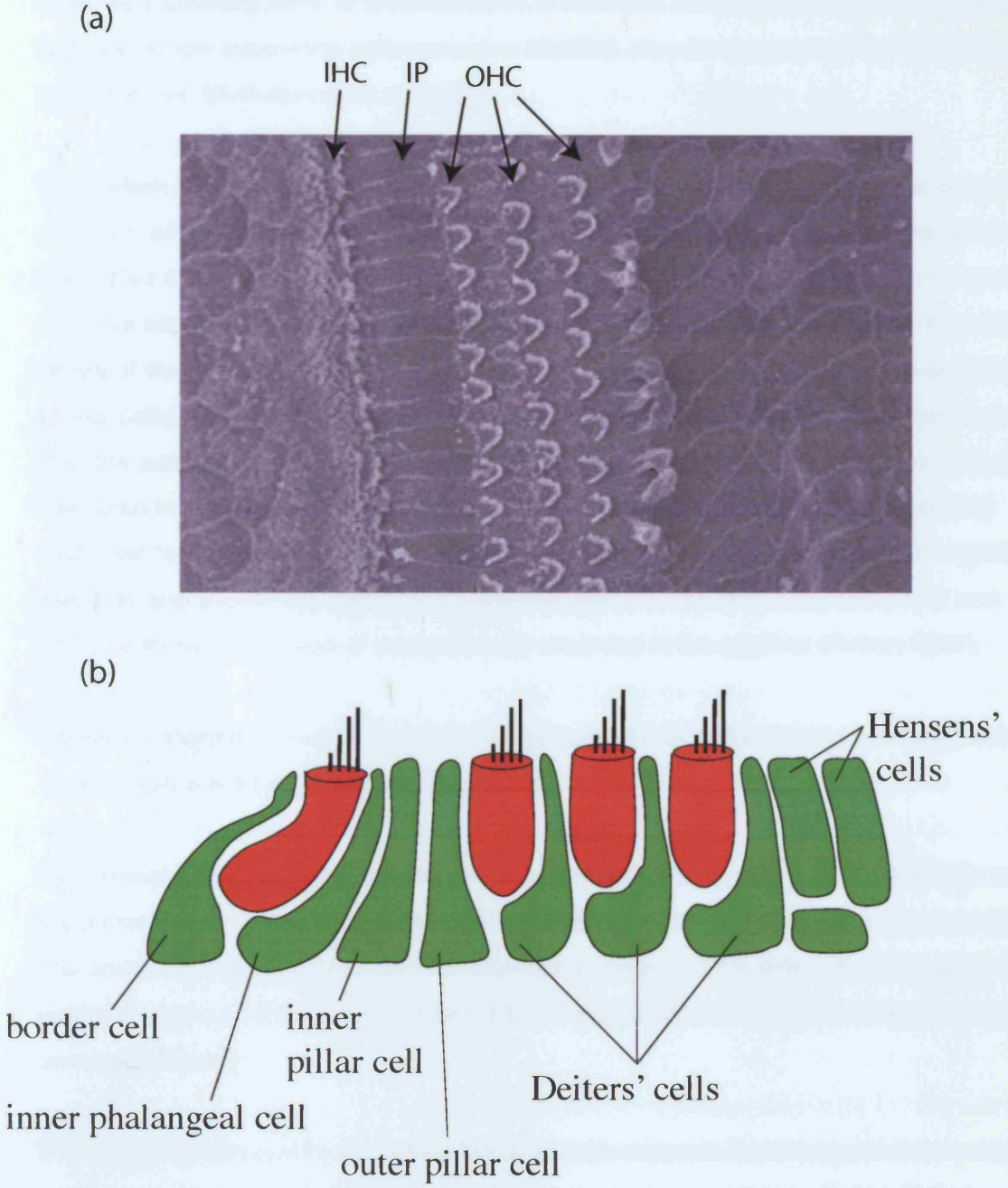


Figure 1.5
Mature structure of the organ of Corti in the mouse cochlea.

(a) SEM showing inner hair cells (IHC), inner pillar cells (IP) and outer hair cells (OHC) in surface view of the adult organ of Corti.
(b) Schematic showing the types of supporting cells present in the organ of Corti, with hair cells shown in red, and supporting cells in green.

labelled several days before their terminal mitosis, they gave rise to clones that contained either all supporting cells, or a combination of hair cells and supporting cells. Single hair cells and single supporting cells were also labelled, as were supporting-cell and hair-cell pairs (Fekete, Muthukumar et al. 1998).

The pattern of hair cells and supporting cells is less elaborate in the vestibular sensory epithelia, where the epithelium forms patches with an alternating mosaic of the two cell types. Cell differentiation in the vestibular patches has not been studied in as much detail as in the cochlea. In the maculae, a wave of differentiation occurs, spreading from the centre of the patch outwards. Hair bundles are first seen in the utricle in mice at E13.5. A similar pattern of hair cell development is seen in the cristae, which develop slightly earlier than the maculae (Lim and Anniko 1985; Sans and Chat 1982). Immature hair cells are also seen in the central area of the maculae at later stages. The extended period of vestibular hair cell production is in agreement with the pattern of mitoses. The majority of hair cells and supporting cells in the vestibular patches are born between E13.5 and E17.5, with no sharp peak of production as observed in the cochlea (Ruben 1967).

1.5 Notch signalling may establish the mosaic of hair cells and supporting cells through a process of lateral inhibition

The sensory epithelium of the inner ear is composed of mechanosensory hair cells and supporting cells arranged in an alternating pattern, so that hair cells are separated from one another by supporting cells. This alternating pattern of the different cell types in itself suggests that a mechanism of lateral inhibition might underlie the patterning of cells in the sensory epithelium.

The pattern of expression of several Notch ligands supports this theory. In the nervous system, expression of *Delta1* is seen in nascent neurons; correspondingly, *Delta1* expression in the ear occurs in nascent hair cells in a salt and pepper mosaic with the Delta-negative, non-sensory supporting cells.

Thus, by analogy with other systems, the theory for the ear is that a group of cells, all capable of forming hair cells, all initially express Delta and Notch, and deliver Notch activation to one another in a competitive fashion. Small differences in the ability of neighbouring cells to deliver or to receive Notch signalling would then be amplified by a

process of lateral inhibition. Activation of Notch in the inhibited cells causes them to downregulate expression of Delta, enabling their neighbours to escape inhibition, upregulate Delta, and differentiate as hair cells. The salt and pepper pattern of Delta expression and hair-cell differentiation would be established in this way.

1.5.1 Evidence for the role of Notch signalling in hair cell patterning in zebrafish

The most compelling evidence that Notch mediated lateral inhibition determines hair cell versus supporting cell fate decisions in the sensory patches of the ear comes from studies in zebrafish. The *mindbomb* (*mib*) mutant was isolated in a screen for zebrafish with defects in brain development. Homozygous *mib* mutants exhibit early and excessive neurogenesis, similar to the neurogenic phenotype seen in Notch pathway mutants in Drosophila (Jiang, Brand et al. 1996; Schier, Neuhauss et al. 1996). This excessive production of neurons occurs at the expense of late cell types such as radial glia.

Analysis of ear development in *mib* mutants reveals a similar defect in cell fate decisions in the sensory epithelium, with hair cells being produced early and in excess, at the expense of supporting cells. The hair cells subsequently die and are extruded from the epithelium (Haddon, Jiang et al. 1998; Haddon, Mowbray et al. 1999). *Mindbomb* has since been shown to encode an E3 ubiquitin ligase which ubiquitinates the intracellular portion of Delta, leading to its internalisation. This is thought to be required for efficient activation of Notch by Delta (Itoh, Kim et al. 2003). Specification of the cells that form the sensory patch does not appear to be reduced in the *mindbomb* mutant, suggesting that mindbomb is required for lateral inhibition alone, and not in establishing the prosensory patch.

There are at least eight Notch ligands in zebrafish; five Deltas, and three Serrate genes. *DeltaA*, *DeltaB, DeltaC* and *DeltaD*, and also *SerrateB* are expressed in hair cells as they develop (Haddon, Jiang et al. 1998; Smithers, Haddon et al. 2000). Zebrafish homozygous for a dominant negative form of one of the Delta ligands, $DeltaA^{dx2}$, display a mild version of the *mindbomb* phenotype: an increase in hair cell numbers, accompanied by a decrease in supporting cell numbers (Riley, Chiang et al. 1999). This suggests that the multiple Notch ligands expressed in the nascent hair cells act redundantly to mediate lateral inhibition.

1.5.2 Evidence for the role of Notch signalling in hair cell patterning in chick

Experiments in chick and in mouse have produced results that are more difficult to interpret than the zebrafish data. While there is persuasive evidence that Notch signalling mediates lateral inhibition in the central nervous system, controlling the production of neurons, its role in ear development in birds and mammals appears more complex.

The role of *Delta1* in chick ear development was investigated using retrovirally-mediated ectopic expression of *Delta1*. This did not have the expected effects upon cell type specification. Ectopic expression of *Delta1* in the immature sensory patch did not, as might have been expected, detectably inhibit hair cell differentiation: hair cells were produced in proportion to supporting cells and in the normal pattern (Eddison, Le Roux et al. 2000). From this result, it appears that *Delta1* alone is not able to determine hair cell versus supporting cell fate decisions, although, as I shall discess at the end of chapter 5, another interpretation is possible. Delta1 does appear to play some role in hair cell patterning, however, and has been shown to be re-expressed during hair cell regeneration in the auditory epithelium in chick (Stone and Rubel 1999).

More clear evidence for the role of Notch signalling in hair cell patterning was seen upon ectopic expression of the activated form of Notch, the Notch intracellular domain (N^{ICD}) (Daudet and Lewis in press). Transient ectopic expression of N^{ICD} was achieved by electroporating plasmid containing the N^{ICD} coding sequence downstream from a constitutive promoter into the otic vesicle. Cells ectopically expressing the construct were visualised by the addition of a GFP or HA tag to the NICD. As predicted by the lateral inhibition model, ectopic expression of N^{ICD} within the developing sensory patches inhibited hair cell production. In groups of cells expressing N^{ICD} within sensory patches, 1.2% of cells developed as hair cells, compared to 16.7% of cells in control patches. This is a striking confirmation of the role of Notch-mediated inhibition in hair cell patterning during development of the vertebrate inner ear.

1.5.3 Notch signalling is also involved in specifying sensory patches

Ectopic expression of activated Notch in chick embryos has also revealed an additional role of Notch signalling in initiating the development of sensory patches, as well as patterning the hair cells within them (Daudet and Lewis in press). Groups of cells ectopically expressing N^{ICD} outside the normal sensory epithelia of the ear were sometimes found to form ectopic sensory patches expressing *Serrate1* and containing both hair cells and supporting cells. These ectopic sensory patches were more commonly formed by groups of cells ectopically expressing N^{ICD} that were in proximity to the normal sensory patches. The ectopic patches which formed quite separately from the normal patches sometimes induced the formation of outpouchings of the otocyst to surround them, perhaps demonstrating that the process by which the gross structures housing the sensory epithelia are normally formed during development occurs secondarily to the formation of the sensory patches themselves. Because expression of N^{ICD} in these experiments is transient, it is possible to produce the early effect (induction of prosensory patch) without the late effect (inhibition of hair cell differentiation).

1.6 Disruption of components of the Notch pathway and its target genes results in defects in hair cell patterning in the mouse and rat

Here, I describe what is known of the expression pattern of different members of the pathway and their target genes during normal development, and review the defects in inner ear development seen upon disrupting the function of each.

1.6.1 *Delta1* is expressed in developing hair cells, but the effect of loss of *Delta1* on ear development has not been described

Delta1 homozygous null mice die midgestation, before hair cell patterning has occurred. The effect of loss of *Delta1* on hair cell production has therefore not been studied in mice.

However, there is good information about the *Delta1* expression pattern. This has ben described in $Delta1^{LacZ}$ heterozygous mice, in which one copy of *Delta1* has been replaced by the β-gal coding sequence (Morrison, Hodgetts et al. 1999). X-Gal staining revealed

expression of *Delta1* in the neurogenic region of the otocyst at E10.5, when it marks the delaminating neuroblasts. Subsequently, *Delta1* expression is detected in hair cells of the developing vestibular sensory patches as early as E12.5, and is seen in hair cells of all vestibular patches by E14.5. In the cochlea, likewise, expression is restricted to hair cells; *Delta1* is expressed in inner hair cells from ~E14.5, and spreads gradually to the outer hair cells. This pattern of expression reflects the pattern of hair cell differentiation.

Delta1 is not expressed at detectable levels in the population of prosensory cells, arguing against a role in specifying hair cells as described in the lateral inhibition model. However, it is possible that low, but functionally significant levels of *Delta1* expression occur in a more widespread pattern. In fact, there has been some difficulty in detecting *Delta1* expression and it has previously been reported to be absent from the developing inner ear (Lewis, Frantz et al. 1998). More careful analysis revealed that expression in the hair cells is transientt (Morrison, Hodgetts et al. 1999). No obvious defects in hair cell production were seen in the $Delta1^{LacZ}$ heterozygotes, in either the auditory or the vestibular sensory patches. However, a detailed analysis was not conducted and subtle defects in hair cell production may have been overlooked.

1.6.2 Excess hair cells are produced in the *Jagged2* knockout cochlea

The inner ear phenotype of loss of *Jagged2* has been described in $Jagged2^{DSL/DSL}$ mice, in which the DSL domain of *Jagged2* has been removed. In contrast to *Jagged1* or *Delta1* knockout mice, these *Jagged2* homozygous knockout mice mutants survive through embryogenesis, but die at birth from difficulty in breathing due to severe craniofacial defects. They also exhibit syndactyly (fusion of the digits) in both the hind- and forelimbs (Jiang, Lan et al. 1998). Like *Delta1* (but unlike *Jagged1*), *Jagged2* is expressed in the cochlea in the developing hair cells. Expression is first seen in a narrow band of cells in the basal part of the cochlea, apparently marking the inner hair cells as they begin to differentiate. Expression is detected in hair cells more apically and in the outer hair cell population as differentiation progresses.

As with *Delta1*, *Jagged2* expression is not detected before the beginning of hair cell differentiation, arguing against a role in singling out hair cells from a population of equivalent cells. However, loss of Jagged2 alters the production of cells, which are generated in excess in the cochlea of the knockout mice. An increase in the number of

inner hair cells is seen, and areas of the organ of Corti contain four rows of outer hair cells, instead of the usual three (Lanford, Lan et al. 1999). This pattern of hair cells was seen at E18. No alteration in the timing of hair cell differentiation or maturation was reported, but probably would not have been noticed at this stage of development. There was an increase in the number of hair cells in contact with one another in the *Jagged2* knockout cochlea, suggesting that the excess hair cells were produced at the expense of supporting cells. This was offered by Lanford et al (1999) as evidence for a loss of lateral inhibition. However, hair cells in contact with one another are occasionally seen in the normal cochlea. It appears that the regular pattern of hair cells and supporting cells is in part a result of cell rearrangements during development (Goodyear and Richardson 1997).

Lanford et al (2000) performed further analysis of the *Jagged2* mice, describing the expression of *Math1* and of *Hes5*, two genes that lie downstream of the Notch pathway. *Math1* expression was detected in the extra hair cells that are seen in the *Jagged2* knockout at E17.5. Expression of *Hes5* was dramatically altered in the *Jagged2* knockout cochlea, being either undetectable or at very low levels compared to the wildtype situation at E17.5, supporting the idea that Jagged2 signals to neighbouring cells, inhibiting their expression of Notch regulated genes (Lanford, Shailam et al. 2000).

The effects of loss of *Jagged2* are modified – though only slightly - when *Lunatic fringe* (*Lfng*) is also lost (Zhang, Martin et al. 2000). *Lfng* is expressed in those supporting cells that are in direct contact with the hair cells - the inner phalangeal cells, the outer piller cells and the Deiter's cells. There is no apparent disruption of cell fate patterning in the homozygous *Lfng* knockout cochlea, where both copies of *Jagged2* are present. However, loss of one or both copies of *Lfng* on a *Jagged2* knockout background diminishes the overproduction of inner hair cells in a dosage-dependent manner, though the extra row of outer hair cells is still present. It is not clear why the effect of loss of *Lfng* is restricted to the inner hair cell population.

1.6.3 Loss of inhibitory bHLH genes results in excess hair cell production

Mammals have several *Hairy/Enhancer of Split* homologues, named *Hes* genes. Loss-of-function mutations in two of these, *Hes1* and *Hes5,* result in increased numbers of hair cells in both auditory and vestibular sensory patches. In the cochlea, loss of *Hes1* mainly affected inner hair cells, while loss of *Hes5* mainly affected outer hair cells.

In the *Hes1* null cochlea, inner hair cell "doublets" are seen at late embryonic and early postnatal stages, with over a third of the cochlea exhibiting an incomplete extra row of inner hair cells at P0 (Zheng, Shou et al. 2000; Zine, Aubert et al. 2001). A mild increase in the number of outer hair cells was also seen in these mice, though this effect was minor compared to the effect upon inner hair cells. Conversely, in the *Hes5* null cochlea, a complete extra row of outer hair cells was observed along almost half the length of the organ of Corti at P0, with only a few extra inner hair cells.

Hair cell production in the absence of both *Hes1* and *Hes5* has not been studied as double homozygous knockout mice die at E11.5 (Ohtsuka, Ishibashi et al. 1999). However, double mutant mice, carrying only one copy of either *Hes1* or *Hes5*, survive to birth and production of auditory hair cells in these mice has been described. Loss of one copy of *Hes5* on a *Hes1* null background (*Hes1-/-;Hes5+/-*) further increased the number of inner hair cells produced, so that inner hair cell duplications were seen in most parts of the organ of Corti along its apicobasal length, and the number of outer hair cells was also increased. Likewise, there was an increase in outer hair cell numbers in the *Hes5* null mutant when one copy of *Hes1* was also lost (*Hes5-/-;Hes1+/-*), in addition to an increase in inner hair cell numbers. This suggests that while the two Hes genes differ in their relative importance in production of different hair cell types in the organ of Corti, the action of both Hes genes is required for the normal patterning of both inner and outer hair cells.

The expression patterns of *Hes1* and *Hes5* do not obviously reflect their differing effects upon inner versus outer hair cells. Transcripts of both genes can be detected in the developing inner ear from E13.5 (Zheng, Shou et al. 2000; Zine, Aubert et al. 2001). However, the majority of expression analysis of these genes has been performed on late embryonic and early postnatal stages, long after the patterning of hair cells has taken place. At E17.5, *Hes1* is expressed in supporting cells of the utricle, and in the non-sensory greater epithelial ridge (GER) and lesser epithelial ridge (LER), which lie adjecent to the organ of Corti. Little staining was seen within the organ of Corti itself. At this stage, *Hes5* expression is seen in both the LER, and Deiters' cells, Pillar cells and inner phalangeal cells (those supporting cells in direct contact with hair cells).

Studies of *Hes* gene expression at intermediate stages are restricted to *Hes5*. It has been reported that in the basal cochlea the onset of *Hes5* expression occurs at E15.5 (Lanford, Shailam et al. 2000). At this stage, expression of *Hes5* is restricted to a narrow band of cells. As development proceeds, the domain of expression broadens, matching the timing of differentiation of inner, then outer hair cells. Interestingly, expression is downregulated in the basal part of the cochlea by E17, but is observed in the apex of the cochlea into adulthood.

Turning from the cochlea to the vestibular region, one finds that at E13.5 *Hes5* is expressed in three patches that correspond to the immature cristae. Expression is maintained in these patches until late embryonic stages, when expression levels drop. *Hes5* is expressed throughout the two layers of the sensory epithelium of the cristae initially, then becomes restricted to the supporting cell population. No expression of *Hes5* was reported in the saccule or utricle during the process of hair cell patterning and differentiation. This finding conflicts with data from analysis of the *Hes5* mutant mouse, where an increase in hair cell number is seen both in the saccule and the utricle. It may be that the expression levels of the two *Hes* genes differ in the utricle and the saccule, and that lower – but functionally significant - levels in the maculae could not be detected by in situ hybridisation.

1.6.4 Early loss of *Notch1* results in excessive hair cell production

Expression of *Notch1* in the ear has been studied by in situ hybridisation (Lanford, Lan et al. 1999). *Notch1* has a broad domain of expression in the developing cochlea between E12.5 and E14.5. At this stage, it is seen in the apical part of the epithelium in the region where the sensory patch will form, and throughout the greater epithelial ridge. As hair cells develop, expression becomes restricted to the supporting cells, and is maintained into adulthood (Zine, Van de Water et al. 2000).

Mice homozygous for a knockout mutation of *Notch1* die early in development due to vascular defects (Swiatek, Lindsell et al. 1994; Huppert, Le et al. 2000). Heterozygous individuals, however, are viable. These mice exhibit four rows of outer hair cells, as opposed to the normal three rows, in many parts of the cochlea, resulting in a small increase in the total number of hair cells (Zhang, Martin et al. 2000). No

supernumary inner hair cells were observed in the cochlea from these *Notch1* heterozygotes. Experiments reported at a recent scientific meeting report a more dramatic overproduction of hair cells in mice with a conditional loss of *Notch1* (Kiernan et al., Molecular Biology of Hearing and Deafness, Baltimore, October 2004).

1.6.5 *Jagged1* heterozygotes exhibit defects in inner ear development

Jagged1 is expressed early in development, marking the prosensory patches, and later becomes restricted to supporting cells, as I shall discuss in more detail in relation to my own findings in chapter 3. Although *Jagged1* knockout homozygotes die early in development (Xue, Gao et al. 1999), heterozygous individuals are viable, and their ear phenotype has been described. Two strains of *Jagged1* mutants, *Slalom* (*Slm*) and *Headturner* (*Htu*), were generated in an ENU mutagenesis screen, where they were found to display head-shaking behaviour indicative of inner ear defects (Kiernan, Ahituv et al. 2001; Tsai, Hardisty et al. 2001). Both *Htu* and *Slm* are missense mutations in the sequence coding for the second EGF-repeat in the *Jagged1* gene, a domain of the ligand required for high affinity interactions with the Notch receptor (Shimizu, Chiba et al. 1999).

Mice homozygous for these mutations of the *Jagged1* gene die mid-gestation, exhibiting vascular defects as observed in the *Jagged1*^{dsl} homozygotes, like in transgenic knockout homozygotes. Heterozygotes exhibit truncations of the posterior semi-circular canal, which is usually accompanied by loss of the anterior semi-circular canal. A reduction or loss of the associated ampullae is also observed. In the cochlea, hair cell patterning is disrupted in an unexpected way. The number of outer hair cells is decreased, so that the usual three rows were reduced to one or two rows in many parts of the cochlea. Thus, the number of outer hair cells in the basal region of the cochlea of the heterozygous *Htu* mutants was reduced by 33%. Conversely, inner hair cells, or at least hair cells in the inner region, were produced in excess. The combined effect of increased inner hair cells and decreased outer hair cells was a 16% decrease in total hair cell number in the *Htu* heterozygote organ of Corti. In the *Slm* heterozygotes at least, the abnormality of hair-cell production was more extreme in the basal part of the cochlea than in the apex. The interpretation of these defects in hair cell production will be discussed in more detail later in this thesis.

1.6.6 Blocking *Jagged1* and *Notch1* in vitro causes excessive hair cell production

The later role of Jagged1 and Notch1 in the supporting cells of the sensory patch was investigated by Zine et al (2000), by treating cochlea explants taken from rats aged between E18 and P3 with antisense oligonucleotides against *Jagged1* and *Notch1* (Zine, Van De Water et al. 2000). In both cases, the sensory patch was enlarged. This was largely due to an increase in the number of outer hair cells. A less dramatic effect was seen with inner hair cells, with a maximum of one extra row forming after antisense treatment. The effect of *Notch1* antisense treatment was more dramatic than that seen in the *Jagged1* antisense cochleas, with between 5 and 8 rows forming in the case of *Notch1* (when explanted at E16), compared to 3 to 4 rows in the case of *Jagged1*.

The numbers of supernumary hair cells produced in these explants decreased as the age of mice from which the cochleas were taken increased, with a maximum effect seen in explants taken from embryos at E16, and only a slight effect at P3 (Zine, Van De Water et al. 2000).

1.6.7 The bHLH gene *Math1* is required for hair cell development

Math1, the mammalian homolog of Drosophila proneural bHLH gene *Atonal*, is critical for inner ear development. *Math1* null mice completely fail to produce hair cells (Bermingham, Hassan et al. 1999), although the gross structure of the inner ear appears unaffected, and the sensory patches are morphologically identifiable by TEM and SEM. Development of the inner ear appears to occur as normal in the *Math1* null mouse until the time of hair cell production. Cells of the sensory patch in the cochlea exit the cell cycle in the normal, synchronous fashion. However, while apoptotic cells were not detected in the wildtype cochlea between E14 and E18, apoptotic cells were seen in the *Math1* null cochlea in a pattern that closely matches the basal-to-apical wave of hair cell differentiation (Chen, Johnson et al. 2002). The selection of hair cells thus seems to occur normally in the absence of *Math1*, but this population of cells does not survive, or differentiate.

The pattern of expression of *Math1* has been described using both $Math1^{GFP/+}$ and $Math1^{\beta\text{-}Gal/+}$ transgenic mice, the latter approach being the most sensitive method of detection. This approach has revealed expression at E12.5, several days before hair cell production

in the cochlea, throughout the prospective auditory and vestibular sensory patches in the otic vesicle in *Math1*$^{\beta\text{-}Gal}$ heterozygotes. At E13.5, *Math1* expression is detected in a broad domain of cells in the area of the future sensory patch and in cells of the greater epithelial ridge (Woods, Montcouquiol et al. 2004). The domain of *Math1*-driven *β-Gal* is broader in the apex of the cochlea and narrows towards the base. A similar pattern of staining is seen in the cochlea at E15.5, as hair cells begin to differentiate. In the basal region, where hair cells are beginning to differentiate, *Math1* is expressed at higher levels in nascent hair cells. At this time, staining in the apical part of the cochlea remains more uniform. Eventually it becomes excluded from the supporting cells, but is maintained in the hair cell population (Bermingham, Hassan et al. 1999; Woods 2004). Detection of *Math1* expression using an anti-GFP antibody on heterozygous *Math1*^{GFP} reporter mice is less sensitive, and only reveals expression in the hair cells (Chen, Johnson et al. 2002).

This pattern of staining is consistent with the idea that all cells of the patch are initially capable of forming hair cells, but that hair cells are selected by a process of lateral inhibition, delivered by Notch signalling. Activation of Notch switches on expression of Hes genes, which in turn inhibits *Math1* expression. Thus, the expression of *Math1* becomes restricted from a wide group of cells, all competing to form hair cells, to fewer cells, which are then able to develop as hair cells. It also indicates that specification of hair cells does not correlate directly with the time of their terminal mitosis. Cells of the cochlear sensory patch exit the cell cycle between E13 and E14 in a wave that travels from the apex of the cochlea to the base. The upregulation of *Math1* expression in prospective hair cells occurs later, in a wave travelling in the opposite direction, from the base to the apex of the cochlea, as hair cells differentiate.

Further experiments in which *Math1* was misexpressed in the cochlea in vitro showed that *Math1* is sufficient for hair cell production in non-sensory epithelia in the cochlea (Zheng and Gao 2000: Woods, Montcouquiol et al. 2004). Ectopic hair cells were formed in regions of the non-sensory epithelium of the greater epithelial ridge that misexpressed *Math1*. Another key finding of Woods et al, (2004) is that ectopic *Math1*-induced hair cells are able to induce *Math1*-negative non-sensory epithelial cells to become supporting cells.

1.7 Other factors involved in sensory patch specification

From the outset, expression of Notch ligands within the otocyst is localised, implying that other factors must be operating to define where the Notch pathway is to be activated. There a several factors implicated in sensory patch specification, and I give a brief deascription of some of these below.

1.7.1 Pax-Six-Eya-Dach

The highly conserved Pax (Paired Box) – Six (sino-oculariS) – Eya (eyes-absent) – Dach (dachshund) network of genes is required for the development of several sense organs, During development of the eye, *eyeless* (a Drosophila *Pax6* gene) acts as a master regulator, with ectopic expression inducing the formation of ectopic eyes (Halder, Callaerts et al. 1995). It mediates this function, at last in part, by induction of *sine-ocularis* and *eyes-absent*. A similar network appears to operate to define the location of several sense organs in vertebrates, including the ear.

Eya1 and *Six1* knockout mice exhibit severe defects in ear development, with arrest of ear development at the otocyst stage (Xu, Adams et al. 1999; Li, Oghi et al. 2003; Zheng, Huang et al. 2003; Ozaki, Nakamura et al. 2004). No ear phenotype has been reported for loss of the Dach genes. Analysis of markers of the developing sensory patches revealed a loss of *Lfng* expression in the *Six1* null otocyst, and a reduction in expression of *Bmp4*, indicating a disruption of development of the sensory patches early in development. An increase in cell death and reduction in proliferation was also found in the *Six1* null otocyst, suggesting that *Six1* is normally required for survival and proliferation of cells in the sensory patches (Zheng, Huang et al. 2003; Ozaki, Nakamura et al. 2004). This phenotype is consistent with the reported expression pattern of *Six1*. Early in development, expression is seen the ventral part of the otocyst at E9.5-E10.5. Expression is maintained in all sensory patches between E12.5 and E14.5 (Zheng, Huang et al. 2003; Ozaki, Nakamura et al. 2004).

Less dramatic phenotypes have been seen in mice carrying mutations of those Pax genes expressed in the developing ear epithelium - *Pax2* and *Pax8*. *Pax2* is expressed in the

medial wall of the otocyst, where it overlaps partially with the expression of *Lfng* marking the immature sensory patches of the utricular macula and saccular macula/cochlea. Reports vary as to whether it is also expressed in the sensory patches throughout their development, but expression has been found in differentiated hair cells (Lawoko-Kerali, Rivolta et al. 2002; Burton, Cole et al. 2004). *Pax2* null mice exhibit a severe reduction in the size of the cochlea, of which only half a turn is present (Torres, Gomez-Pardo et al. 1996; Burton, Cole et al. 2004). *Pax8* displays a similar expression domain to that of *Pax2*, but does not seem to be required for ear development (Xu, Adams et al. 1999).

The Pax genes are particularly relevant to this study as there is some evidence that they may interact with Notch signalling in sensory organ development. In Xenopus, ectopic Notch activation resulted in the formation of ectopic eye structures, which expressed *Pax6* (Onuma, Takahashi et al. 2002). Also, the zebrafish Pax2.1 mutant displays a mild neurogenic phenotype in the ear, suggesting that it is required in some way in lateral inhibition (Riley, Chiang et al. 1999).

1.7.2 Dlx

The *Dlx* genes, vertebrate homologs of the Drosophila *Distal-less* gene, are also involved in ear development. Experiments in zebrafish have shown that otic placode formation requires the activity of two *Dlx* genes, *dlx3* and *dlx7*: knockdown of both genes using antisense morpholinos resulted in the formation of much reduced otocysts (Merlo, Paleari et al. 2002; Solomon and Fritz 2002). Analysis of gene expression in morpholino injected fish indicated that the dlx genes act downstream of the zebrafish *pax8* gene in otocyst development, but are required for normal *pax2.1* expression. Further information about the requirement for dlx genes in ear development comes from the *Dlx5* mouse mutant. *Dlx5* is expressed in the dorsal part of the otocyst, in the area of the developing cristae. *Dlx5* null mice have severe defects of the vestibular apparatus, exhibiting a loss of the anterior and posterior semicircular canals, and a reduction in the horizontal canal. Expression of *Bmp4* is lost in the *Dlx5* null otocyst indicating a loss of the developing cristae, though the expression of *Lfng*, marking the developing maculae and cochlea, is unaffected (Acampora, Merlo et al. 1999; Merlo, Paleari et al. 2002). *Dlx5* thus appears to be specifically required for development of the cristae.

1.7.3 Fibroblast Growth Factors

Fibroblast growth factors (FGFs) have also been implicated in patterning of the sensory patches. Conditional inactivation of *Fgfr1* (*Fgf receptor-1*) in the developing ear causes a loss of hair cells, resulting in the formation of small islands of hair cells along the length of the cochlea, instead of the normal four orderly rows. A defect in cell proliferation was observed in the cochlea of these mice, suggesting that the defect in hair cell patterning is due to failure of expansion of the prosensory patch. Another Fgf receptor, Fgfr3, is required specifically for the production of pillar cells in the developing organ of Corti (Colvin, Bohne et al. 1996; Pirvola, Ylikoski et al. 2002).

1.7.4 Bone Morphogenetic Proteins

As I have described, *Bmp4* is expressed in the developing cristae early in development, and is later found in both maculae and in the cochlea. A requirement for Bmps in ear development has been demonstrated by treatment of the developing chick ear with the Bmp4 antagonist, noggin. Noggin caused gross defects of the semicircular canals, with a varying loss of the three canals and their ampullae. Loss of the semicircular canals corresponded with loss of the ampullae, and of the cristae. Bmps have since been implicated in canal genesis, and maintained by Fgf signalling from the sensory patches (Chang, Brigande et al. 2004).

1.7.5 $p27^{Kip1}$

Progression of a cell through the cell cycle is controlled by a core group of proteins: the cyclins and the cyclin-dependent kinases (Cdks). The activity of cyclin/Cdk complexes is modulated by a group of inhibitory proteins, the cyclin-dependent kinase inhibitors (CKIs). In vertebrates, there are two families of CKIs; the Ink4 family ($p15^{Ink4\ B}$, $p16^{Ink4A}$, $p18^{Ink4C}$ and $p19^{Ink4D}$), and the Kip/Cip family ($p27^{Kip1}$, $p57^{Kip2}$, $p21^{Cip1}$). The members of these families differ in that Ink4 inhibitors act specifically to inhibit CDK4/CDK6, whereas Kip/Cip family members act upon a wide variety of cyclin-CDK complexes. Mice with a homozygous loss of $p27^{Kip1}$ exhibit hyperplasia of multiple organs, including the sensory epithelium of the cochlea (Fero, Rivkin et al. 1996; Kiyokawa, Kineman et al. 1996; Nakayama, Ishida et al. 1996).

Both hair cells and supporting cells are produced in excess in the organ of Corti in these mice (Chen and Segil 1999; Lowenheim, Furness et al. 1999). The resulting pattern of four rows of outer hair cells and two rows of inner hair cells is, superficially at least, similar to the *Jagged2* knockout phenotype described above, and to the phenotype seen upon disruption of both *Hes1* and *Hes5*. This excessive production of hair cells has been attributed to an increase in proliferation of progenitor cells in the absence of $p27^{Kip1}$. Cells of the organ of Corti continue to proliferate at late embryonic stages in the $p27^{Kip1}$ knockout mouse. At P6 there is an increase in the number of cells in the inner pillar cell region. This is also seen in adult mice (4 months), with the additional patterning defect of extra Deiter's cells surrounding the first row of outer hair cells (Chen and Segil 1999; Lowenheim, Furness et al. 1999).

The abnormality in proliferation in the organ of Corti is consistent with the normal expression pattern of $p27^{Kip1}$in this tissue: it is first seen between E12.5 and E13.5 in the cells of the prospective sensory patch as they prepare to exit the cell cycle in synchrony. This domain of $p27^{Kip1}$ staining has been referred to as the zone of non-proliferating cells (ZNPC) (Chen, Johnson et al. 2002). This is in agreement with the classical account of the timing of terminal mitosis of cells of the sensory patch (Ruben 1967). A day later, at E14.5, the first signs of *Math1* expression (as deteced by the $Math1^{GFP}$ reporter mice) are seen in the medial border of the ZNPC. Expression of $p27^{Kip1}$ is lost in hair cells as they develop, but is maintained in the supporting cell population until at least the first week after birth (Lowenheim, Furness et al. 1999).

Another CKI implicated in the normal development of the organ of Corti is $p19^{Ink4D}$. Loss of $p19^{Ink4D}$ does not affect the normal patterning of hair cells in the cochlea, with homozygous null mice exhibiting the normal pattern of four rows of hair cells at postnatal stages. However, within two weeks of being born, these mice exhibit a progressive loss of hair cells, due to the re-entry of these cells into the cell cycle and their subsequent cell death (Chen, Zindy et al. 2003). On the basis of these results it appears that two CKIs are required for the maintenance of the postmitotic state of cells in the cochlea, with $p27^{Kip1}$ being required for cell cycle exit of all cells of the patch early in development and the maintenance of a quiescent state of supporting hair cells into adulthood, and $p19^{Ink4D}$ being required specifically for maintaining hair cells in a postmitotic state after development of the sensory patch is complete.

The overproduction of hair cells in the $p27^{Kip1}$mutant would appear to occur due to deregulation of proliferation of cells in the developing organ of Corti. However, the same defect in hair cell production seen in mice with a disruption of the Notch signalling pathway, by mutation of *Jagged2*, *Hes1* or *Hes5*, is most likely due to a defect in lateral inhibition.

1.8 Aims and scope of this work

The aim of this study was to investigate the function of two Notch ligands, Delta1 and Jagged1, in development of the sensory epithelia of the inner ear. Both ligands were known to be expressed in this tissue during its development, but their respective roles in ear development were not clear.

Mice homozygous for knockout mutations of Notch receptors and ligands die around midgestation, before patterning of the inner ear sensory epithelium takes place. In order to investigate the effect of loss of Notch ligands *Delta1* and *Jagged1*, I have used the Cre-LoxP system to produce mice with a tissue specific loss of these genes. I have found that *Delta1* and *Jagged1* play strikingly complementary roles in the development of the mammalian inner ear.

I begin by describing the dynamic pattern of Jagged1 distribution in the developing cochlea as hair cells are produced. I then present analysis of hair cell production in the vestibular sensory patches and in the cochlea of *Jagged1* conditional knockout mice. This reveals a dramatic reduction in the size of the auditory sensory epithelium, and a loss of vestibular patches, primarily affecting the cristae. This is in agreement with the proposed role of *Jagged1* in specifying sensory patches, and argues against a role in preventing premature differentiation. Unexpectedly, I have also found that expression of $p27^{Kip1}$ is lost or severely reduced in the *Jagged1* conditional knockout cochlea.

Secondly, I describe defects in inner ear development seen in *Delta1* conditional knockout mice. Loss of Delta1 causes a deficiency of maculae, but exessive production of hair cells in the cochea. Surprisingly, the gain of hair cells is not accompanied by a loss of supporting cells, as would be predicted by the lateral inhibition hypothesis, but rather by a gain of supporting cells. The abnormality in hair cell production is superficially similar to

that seen in the cochlea of $p27^{Kip1}$ null mice, though no disruption of $p27^{Kip1}$ or of proliferation in the developing cochlea is found, and the machanism, as I shall explain, is probably different.

Thus, *Delta1* and *Jagged1* have roles in the development of the mammalian inner ear that seem to mirror one another. While loss of *Delta1* causes an expansion of the auditory sensory patch and a loss of maculae, loss of *Jagged1* causes a reduction of the auditory sensory patch and a loss of cristae. I shall discuss how these contrasting effects can be interpreted in terms of the early inductive and late inhibitory actions of Notch.

Chapter 2

Materials and Methods

2.1 Mouse breeding strategy

All animals were housed at Clare Hall laboratories, and technicians performed all timed matings and were responsible for animal care. I was responsible for managing the colony, for genotyping all strains of mice used, and for collection and processing of all embryos used in this study.

The same breeding strategy was adopted for the generation of both *Jagged1* and *Dll1* conditional knockout mice. The goal was to produce conditional knockout mice that were homozygous for the floxed allele, and heterozygous for *Foxg1-Cre*. Thus, in the case of the *Jagged1* conditional knockout mice, I wanted to generate mice that carried two copies of the *Jagged1*^{flox} allele, *Jagged1*$^{flox/flox}$, and one copy of *Foxg1-Cre* (*Foxg1*$^{Cre/+}$). In a mouse of this genotype both copies of *Jagged1* would be removed in the regions of *Foxg1-Cre* expression. I also required heterozygous *Foxg1-Cre* mice as control littermates, as the *Foxg1-Cre* is a knockout allele of *Foxg1*. Loss of *Jagged1* in the conditional knockout mice thus occurs on a *Foxg1* heterozygous knockout background.

Pregnancies containing embryos with the above genotypes were generated by crossing stud male mice that were doubly heterozygotes for *Foxg1-Cre* and *Jagged1*^{flox} (*Jagged1*$^{flox/+}$; *Foxg1*$^{Cre/+}$) with *Jagged1*$^{flox/+}$ females in timed matings. The litters from parents with these genotypes are predicted to contain homozygous conditional knockout mice (*Jagged1*$^{flox/flox}$; *Foxg1*$^{Cre/+}$) at a frequency of one in eight.

2.2 Genotyping by PCR

Genomic DNA was isolated from tailsnips from mice at stages from E15.5 embryos to adult mice, and from yolk sacs from younger embryos. These tissue samples were digested in a proteinase K buffer overnight at 60°C, and tail homogenates were diluted with sterile H_2O to a final volume of 700μl. 400μl of this was transferred to a clean Eppendorf tube, and 1ml of absolute ethanol was added to precipitate the DNA. The tube

was inverted several times to mix, then centrifuged. Pellets containing DNA were washed with 70% ethanol, air dried, then resuspended in 100µl sterile H_2O. 1µl of this final DNA solution was used for each PCR reaction.

All transgenic mice used in this study were genotyped by PCR, using the Taq PCR kit from Qiagen, as per manufacturer's instructions. Details of the primers used for each mouse line, are given in the table below, and in the relevant results chapters. PCR reaction products were separated by electrophoresis on 1% agarose gels containing ethidium bromide, and photographed under UV light.

Proteinase K buffer.
100µl 5M NaCl, 50µl Tris pH7.5, 5µl 0.5M EDTA, 500µl 10% SDS, 100µl Proteinase K (20mg/ml), made up to 5ml with H_2O.

Mouse line	5' primer	3' primer
Jagged1^{flox}	5Jspe TGAACTCAGGACAGTGCTCT	3Jspe GTTTCAGTGTCTGCCATTGC
Jagged1$^{\Delta flox}$	5Jspe TGAACTCAGGACAGTGCTCT	23JSma ATAGGAGGCCATGGATGACT
Jagged1^{flox} 3' LoxP site	SeqJdg1d GCCTCCTAGCCAGACAACAGG	23JSma ATAGGAGGCCATGGATGACT
Jagged1$^{\Delta flox}$ (RT-PCR)	RB11 AGTTTCGCCTGGCCGAGGTCCTA	RB14 AGTTGGTCTCACAGAGGCAC TGC
Delta1 ^{flox}	5XhoD CACACCTCCTACTTACCTGA	3XhoD GAGAGTACTGGATGGAGCAA G
Delta1$^{\Delta flox}$	5DXho1 CACACCTCCTACTTACCTGA	3DEcoRV GGCGCTCAAAGGATATGGGA
Cre	Cre5' GGACATGTTCAGGGATCGCCAGGCG	Cre3' GCATAACCAGTGAAACAGCA TTGCTG

2.3 RT-PCR

Total RNA was isolated from the upper half of E10.5 embryos, including the forelimb. The lower half of these embryos was used to isolate genomic DNA for genotyping by PCR.The upper halves of the embryos were placed in Eppendorf tubes and frozen on dry ice. They

were then stored overnight at 70°C to allow time for genotyping to be performed. The embryos were then defrosted, and the tissue was macerated in the Eppendorf tube.

Total RNA was then isolated from these samples using the Promega SV Total RNA Isolation System, as per manufacturer's instructions. 1µg of this RNA was then used as template for reverse transcription to produce cDNA (RETROscript Kit, Ambion). Primers RB11 and RB14 were used to amplify the RNA transcript produced from the recombined allele. RB11 spanned exons 2 and 3, and thus specifically recognises spliced RNA.

2.4 Sequencing

To check the LoxP sites in the *Jagged1*^{flox} mice I sequenced PCR products generated from genomic DNA from homozygous individuals, using primers flanking each LoxP site. For amplifying and sequencing the 5' LoxP site the primers were 5JSpe and 3JSpe, and those for amplifying and sequencing the 3' LoxP site were SeqJgd1b and 23JSma. All primers were annealed at 58°C. PCR products were isolated by after being run on an agarose gel using the QIAGEN QIAquick Gel Extraction Kit.

Sequencing reactions were set up using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit as per manufacturer's instructions, and the reaction products were isolated by ethanol precipitation. Samples were run on capillary sequencing gels (Prism 3730).

2.5 Collection of Embryos

Pregnant females were killed using CO_2 and cervical dislocation. The uterine horns were removed, and the embryos dissected out. Embryos were staged counting the day of the vaginal plug as E0.5, and this staging was reassessed upon collection according to their appearance (Theiler 1989; Kaufman 1992). Early embryos (E9.5-E14.5) were staged based upon limb development (Martin 1990). Embryos that looked dead or dying were discarded. Embryos aged E9.5 – E12.5 were fixed whole and heads from older embryos were bisected before fixation in 4% formaldehyde in PBS at room temperature for two hours.

2.6 Cryosections

After fixation, specimens to be embedded for cryosectioning were rinsed in PBS, and equilibrated in 5% sucrose in PBS overnight at 4°C. They were embedded in 1.5% Lennox agar (Gibco-BRL) with 3% sucrose, and cut into prism shaped blocks for sectioning. Blocks were then equilibrated in 30% sucrose before freezing on dry ice. Cryosections of 15μm thickness were produced by Jennifer Corrigan, and slides were shipped on dry ice and stored at –70°C.

2.7 Histological staining of paraffin sections

Paraffin sectioning and staining of specimens was performed in the Histology Lab, CRUK. The protocol was as follows. Specimens were fixed for an extended period of time (2-7 days) in 4% paraformaldehyde in PBS at 4°C. They were then rinsed, dehydrated in a graded series of alcohol series, cleared in xylene, embedded in paraffin and sectioned at 4μm. Sections were de-waxed in xylene, rehydrated and stained with Haematoxylin and Eosin

2.8 Synthesis of Riboprobes

A riboprobe against mouse *Notch3* was produced from a plasmid containing base pairs 3340-4250 of the *Notch3* gene, obtained from Michael Lardelli (Williams, Lendahl et al. 1995). Template DNA was prepared by linearisation of this plasmid using BamHI, and RNA was transcribed using the T3 RNA polymerase (Roche, RNA transcription kit).

2.9 In Situ Hybridisation on Cryosections

The pattern of gene expression was determined by in situ hybridisation on cryosections using the method described briefly in Myat, Henrique et al. (1996). Slides were defrosted at room temperature for at least 1 hour. The riboprobe was denatured at 75°C for 10 minutes, then applied to the sections in hybridisation buffer (solutions are listed below) and incubated overnight at 65°C.

After hybridisation of the probe, sections were washed several times in post-hybridisation wash solution at 65°C to remove unbound probe. The slides were then incubated in

blocking solution for 1 hour at room temperature, before application of an alkaline phosphatase-tagged anti-digoxyhenin antibody overnight in the same blocking solution. The coloration reaction was performed in the dark after extensive washing in TBST. Slides were then mounted in a glycerol-based mountant (Citifluor).

Solutions for in situ hybridisation

Hybridisation solution: 50% formamide, 1 x salts, 10% dextran sulphate, 1mg/ml yeast RNA, 1 x Denhardts in H_20.

10X Salts: 114g NaCl, 14.04g Tris Hcl, 1.34g Tris Base, 7.8g $NaH_2PO_4.2H_20$, 7.1g Na_2HP0_4, 100ml 0.5M EDTA up to 1000ml with H_20.

20x SSC: 3M NaCl, 300mM tri-sodium citrate.

Washing solution: 50% formamide, 1 x SSC, 0.1x Tween-20.

TBST: 80ml 1M Tris (pH8.0), 240ml 5M NaCl, 5ml 10% Tween-20, and up to 800ml with H_20.

2.10 Immunohistochemistry on Cryosections

Slides were defrosted at room temperature. Primary antibodies were applied in blocking solution (10% Fetal Calf Serum, 2% BSA, 0.1% Triton X-100 in PBS), and incubated overnight at 4°C. The table below gives details of the primary antibodies used in this study. In the case of the PCNA antibody, an antigen retrieval step was required before application of the antibody. For this, slides were boiled for 10 minutes in 10mM citric acid pH6.0, and cooled at room temperature for 20 minutes. Several washes in PBS were performed before secondary antibodies were applied.

Secondary antibodies used in this study were Alexa Fluor 488 rabbit anti-goat (Molecular Probes A-11008), Alexa Fluor 488 goat anti-rabbit (Molecular Probes) and Alexa Fluor 594 goat anti-mouse (Molecular Probes A-11005). All secondary antibodies were diluted 1:500 in blocking solution and incubated for 1 hour at room temperature. After more washes in PBS, slides were mounted (SlowFade Anti-fade Kit, Molecular Probes), and were imaged with a confocal microscope.

Primary Antibody	Type	Immunogen	Working Dilution.	Reference
Jagged-1 (Santa Cruz)	Goat polyclonal	Synthetic peptide derived from carboxyl terminus of protein	1:100	Morrison et al, 1999.
PCNA (Neomarkers)	Mouse monoclonal	Recombinant rat PCNA protein	1:200	Chen and Segil, 1999.
$p27^{Kip1}$ (Cell Signalling)	Rabbit polyclonal	Synthetic peptide derived from carboxyl terminus of protein	1:100	McKenzie et al, 2004.
Calretinin	Rabbit polyclonal	Recombinant rat calretinin protein	1:1000	Zheng and Gao, 1997.

2.11 Immunostaining of wholemount cochlea

Inner ears were dissected from E15-E17.5 embryos. A small opening was made in the apex of each cochlea before fixation in 4% formaldehyde/PBS for 2 hours at room temperature with agitation. Specimens were given several washes in PBS, and dissected to expose the sensory epithelium in the cochlea.

The tissue was permeabilised in 10% fetal calf serum and 0.3% Triton-X in PBS for 2 hours at room temperature on a shaker to allow penetration of the antibody. The tissue was then immersed in primary antibody diluted in wholemount blocking solution (10% FCS and 0.1% Triton-X in PBS), and was incubated in this overnight at 4°C on a roller. The samples were washed in PBS over the next 6 hours, and secondary antibody was applied at a dilution of 1:500 in wholemount blocking solution for 1 hour at room temperature. Alexa Fluor phalloidin (Molecular Probes) was added with the secondary antibody at 1:100. Specimens were washed several times in PBS, and incubated in a DAPI (Roche) solution for 1 hour. A further dissection was performed before flat-mounting. Cochleas from E17.5 mice were cut into three, giving apical, middle and basal portions. Cochleas from younger embryos were left whole. These were then flat-mounted in Citiflour and imaged with a confocal microscope.

2.12 Confocal imaging and calculating the volume of sensory patches

Fluorescently labelled specimens were imaged using an upright laser scanning confocal microscope (LSM 510, Carl Zeiss Jena). In the case of wholemount cochlea preparations, stacked z-section images were obtained at 2-2.5μm intervals, which encompassed the entire depth of the specimen. A representative image was then chosen for presentation. In the case of sectioned tissue, a single confocal image was acquired for each section of the inner ear. The surface area of the sensory patch in each image (as judged by Jagged1 antibody staining, or the presence of hair cells marked with phalloidin and/or calretinin) was calculated using the "close free shape curve drawing mode" measuring tool (LSM 510 software). The total surface area for each sensory patch was then multiplied by the thickness of sectioned material it was present in (15μm x number of sections in which the patch is seen) to calculate the volume of the patch.

2.13 Counting hair cells and supporting cells

Hair cells were counted using confocal images of wholemount cochlea stained with fluorescent phalloidin. Images were exported from Zeiss LSM 5 Image Browser software with 100μm scale bars and saved in Adobe Photoshop format. 100μm–wide rectangular marquees were drawn using the scale bar as template, and were placed over the region containing hair cells to mark a 100μm length of the developing sensory patch. Hair cells that lay within this zone, and had half or more of their apical surface within the designated area were scored.

Hair cells and supporting cells in the *Delta1* conditional knockout cochlea were counted using z-stacks of images of the wholemount cochlea, which spanned the depth of the sensory epithelium. The cochleas were stained with phalloidin to mark cortical actin, and the actin-rich mechanosensory bundles at the apex of hair cells, with a Jagged1 antibody to mark supporting cells, and with the nuclear dye DAPI, as described above. Hair cells were identified by the presence of a mechanosensory bundle at the apex of the cell, and by the apical location of their nuclei in the sensory epithelium. Supporting cells were identified by their staining positive for Jagged1, and by the basal location of their nuclei in the epithelium.

Each set of images was viewed in Adobe ImageReady, which allowed sequential viewing of the images in each Z-stack. Again, rectangular marquees were drawn to outline a 100μm length of the sensory patch, and outer hair cells and supporting cells that lay within this region, or which had half or more of their cell body within the region, were scored.

2.14 Preparation and imaging of samples using SEM

Cochleas from P0 embryos were processed for scanning electron microscopy by the OTOTO method (Davies and Forge 1987), as follows.

The inner ear was dissected from the temporal bone and small openings were made in the apex and base of the cochlea. The specimens were fixed in a solution of 2.5% glutaraldehyde in 0.1M cacodylate buffer with 3mM $CaCl_2$ at pH7.3 for one and a half hours at room temperature on a rocker. After several short washes in cacodylate buffer the inner ears were left in a solution of EDTA at 4°C for 2-3 days to decalcify.

The cochleas were then coated in osmium by washing in 1% osmium tetroxide in cacodylate buffer for several hours at room temperature. After thorough washing in H_2O, the cochleas were dissected to expose the hair cells, taking care to remove the tectorial membrane that lies in close contact to the hair cells. They were then washed sequentially in 1% buffered Os04 for one hour and 0.5% thiocarbohydrazide (TCH) for 20 minutes, 1% Os04 1 hour, 0.5% TCH 20 minutes, 1% Os04 1 hour, then washed in H_2O. Cochleas were dehydrated through an alcohol series before critical point dying, mounting and sputter coating.

2.15 Paintfilling

Paintfilling of the inner ear was performed as described in Martin and Swanson (1993). The inner ear was dissected out and fixed in 4% paraformaldehyde in PBS overnight or longer. Specimens were dehydrated through a series of methanol solutions and stored at –20°C until required. They were then cleared in methyl salicylate and a solution of 1% white gloss paint in methyl salicylate was injected into the endolymphatic space with a micropipette.

Chapter Three

The Pattern of Jagged1 protein in the developing inner ear

3.1 Introduction

In this chapter I review the published expression data for *Jagged1*, and I add to it my own findings on the distribution of Jagged1 protein in the developing mouse inner ear. This is helpful in trying to understand what *Jagged1* does in the developing ear, and is of relevance to the analysis of the *Delta1* knockout mice, since the functions of the two ligands are intertwined.

The pattern of *Jagged1* expression in the developing mouse ear has been described using in situ hybridisation on wholemount embryos, and on sections (Mitsiadis, Henrique et al. 1997; Morrison, Hodgetts et al. 1999). The earliest reported expression is at E9.5, as a patch in the ventral part of the otocyst. By E12.5, expression is seen throughout all the sensory patches, and in the developing endolymphatic duct. Later, after hair cells have begun to differentiate, *Jagged1* expression is maintained in the supporting cells of the sensory patch, but down-regulated in the hair cells themselves (Morrison, Hodgetts et al. 1999) (although it should be noted that Zine et al report staining with an anti-Jagged1 antibody throughout the floor of the cochlear duct at E16.5, ie. in the nascent hair cells as well as the supporting cells.)

The details of the expression pattern in the cochlea, however, have not so far been adequately described, and are of particular interest in relation to the knockout phenotype. In this chapter, therefore, I give an account of the spatial and temporal pattern of *Jagged1* expression in the cochlea. The cochlea provides an opportunity to look at the pattern of Jagged1 distribution at different stages of hair cell production in one individual, because different stages of sensory patch development are represented at different positions along its apicobasal axis. Wholemount cochlea preparations are used in this study, for the analysis of both *Jagged1* and *Delta1* conditional knockout mice.

3.2 Results

I start by describing the distribution of Jagged1 protein in the early mouse otocyst using immunostaining on cryosections, confirming the results found with in situ hybridisation. I then describe the pattern of Jagged1 distribution in the wholemount cochlea at embryonic stages E14.5 – E17.5.

3.2.1 A patch of Jagged1 positive cells is found in the ventral otocyst at E10.5

Wildtype mice from timed matings were collected at E10.5. The embryos were embedded whole for cryosections, which were taken in the horizontal plane. Slides containing sections of the ear were stained with an anti-Jagged1 polyclonal antibody (Santa Cruz, C-20), and with fluorescent phalloidin. The antibody was raised against a synthetic peptide that mimics the C-terminus of Jagged1, and has been used in previous studies (Morrison, Hodgetts et al. 1999, Chen, Johnson et al. 2002). Staining at E10.5 was at a low level compared to that seen in the late embryonic inner ear, and the protocol had to be adjusted to minimise removal of the stain by repeated washing in PBS (see Material and Methods, section 2.10). Unfortunately, this meant that some residual agar used for embedding the tissue remained, and can be seen as bright specks.

All sections of the otocyst were photographed using a confocal microscope. I then produced a 3-D reconstruction of the whole otocyst. This was done by overlaying traces of the outline of the otocyst from every third section. The zone of Jagged1 staining was marked on these traces. This revealed that Jagged1 protein is restricted to a patch in the ventrolateral part of the otocyst at E10.5 (Figure 3.1).

Staining was also seen with the Jagged1 antibody in the developing eye, with the ciliary margins of the retina and the equatorial zones of the lens staining positive. A set of specimens fixed at E11.5, but analysed less thoroughly, show that by E11.5 the domain of Jagged1 has split into two patches (data not shown).

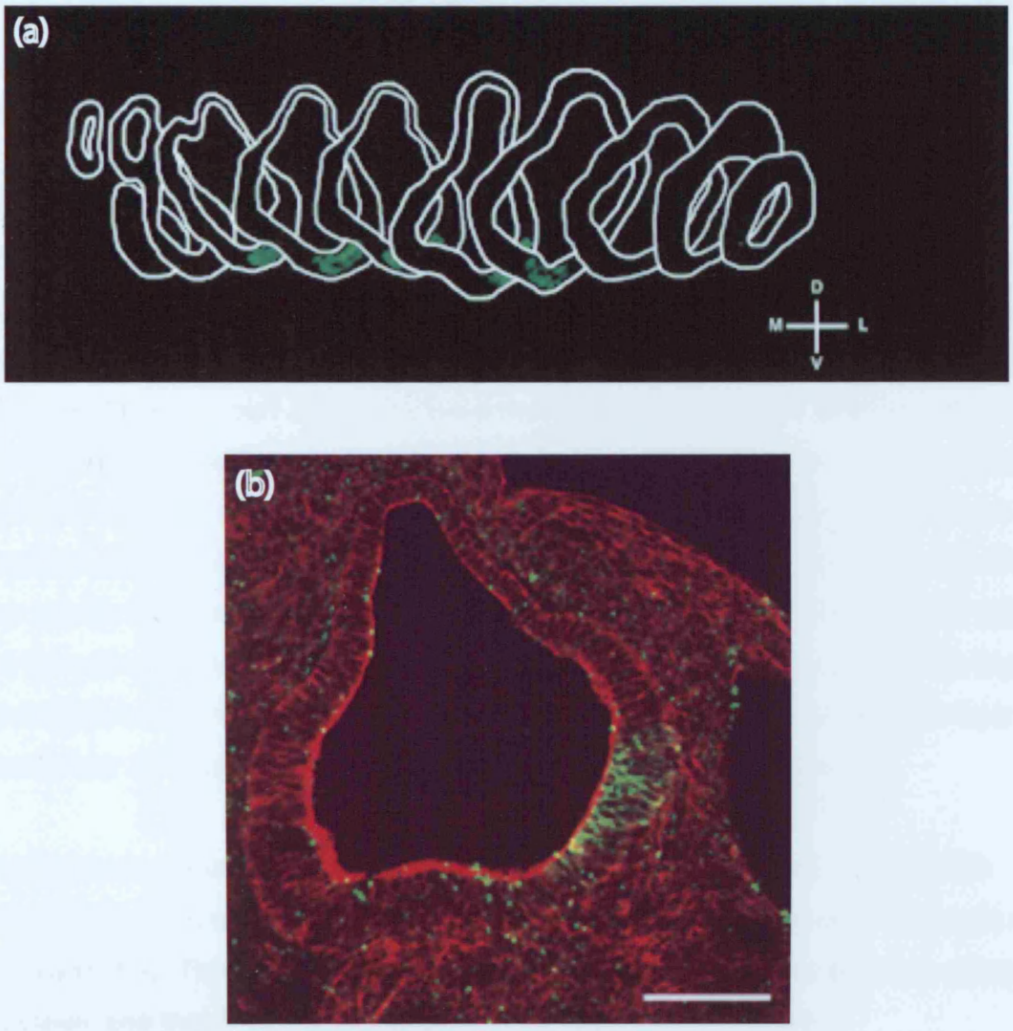


Figure 3.1
Jagged1 distribution in the otocyst at E10.5

Serial sections of the otocyst were stained with Jagged1 antibody (green) and phalloidin (red) (b). These sections were outlined and the area of Jagged staining was highlighted to indicate the position of the Jagged1 patch in the otocyst (a). The orientation indicated in (a) also applies to (b). The section shown in (b) lies between the fifth and sixth sections from the left in (a).
The scale bar in (b) is 100m.

3.2.2 Jagged1 in the wholemount cochlea at E14.5 and E15.5

The pattern of Jagged1 staining in the cochlea at E14.5 and at E15.5 was analysed in wholemounts. At E14.5, Jagged1 staining is seen in a narrow stripe that extends along the apicobasal length of the cochlea (Figure 3.2). The band of staining produced with the Jagged1 antibody has a sharp lateral border (furthest from the central modiolus), but a diffuse medial border. A gradient of intensity of staining exists, with a decrease in intensity more medially.

A day later, at E15.5, the pattern of Jagged1 staining has begun to vary along the length of the cochlea. In order to study this variation in more detail, overlapping Z-series of images from the apex to the base of the E15.5 cochlea were produced. These spanned the depth of the epithelium from the surface to the basement membrane upon which the epithelial cells rest In the apical part of the cochlea a narrow band of Jagged1 positive cells is seen, similar to that described in the cochlea at E14.5 (Figure 3.3). Jagged1 in the middle and basal regions, however, shows an altered pattern of expression (Figure 3.4). As one moves towards the base from the apex, the antibody stain begins to outline a row of cells. These are the inner hair cells, which develop before the outer hair cells. Jagged1 brightly stains the cells that surround these nascent hair cells. This pattern suggests that Jagged1 is downregulated in hair cells as they develop, though double staining using an early marker of hair cells is required to show definitively that this is the case.

Further towards the basal end of the cochlea, the domain of Jagged1 positive cells appears to broaden, with Jagged1 staining seen in the region where outer hair cells will form (Figure 3.4). The staining in this outer region is initially faint, but becomes stronger in more basal, and therefore more differentiated, regions of the cochlea.

3.2.3 Jagged1 in the wholemount cochlea at E17.5

At E17.5, Jagged1 protein is localised to the cell membranes of some of the supporting cells in the organ of Corti, and in the supporting cells of the vestibular patches.

At the lateral border of the sensory patch, Jagged1 protein has a sharp boundary, with bright staining seen in the last row of Deiters' cells, but not in the rows of Hensens' cells, or in the Claudius cells (Figure 3.5). Jagged1 is detected in all supporting cells that are

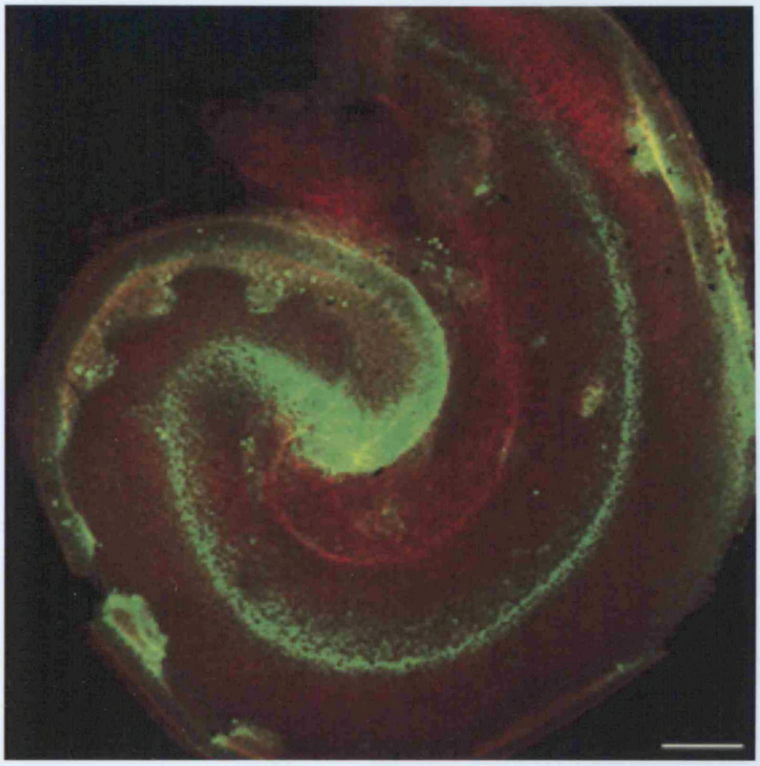


Figure 3.2

Jagged1 distribution in the cochlea at E14.5.

Confocal image of an E14.5 mouse cochlea. The distribution of Jagged1 protein is detected by antibody (green) and actin is stained with phalloidin (red).
Scale bar is 100μm.

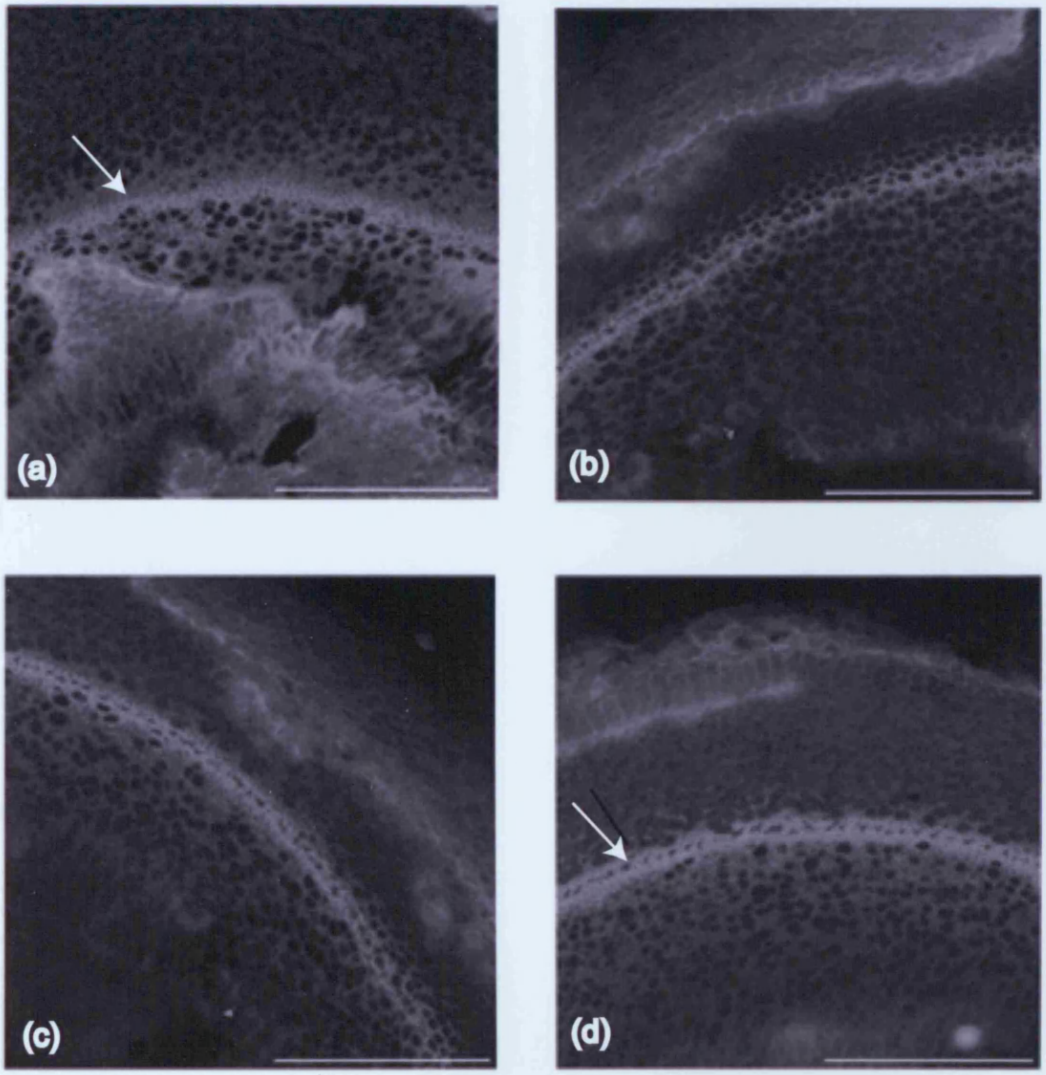


Figure 3.3

Jagged1 distribution in the apical cochlea at E15.5.

Staining with the Jagged1 antibody in the wholemount cochlea at E15.5 reveals a dynamic pattern of distribution as hair cells are produced. In the most apical part of the cochlea (a) a narrow band of cells with low levels of staining is seen (arrow). More basally, the pattern of distribution changes as inner hair cells are formed, (area of inner hair cells indicated by arrow in d) and the level of staining increases (images are from more basal positions from b to d). See also Figure 3.4.
Scale bars are 100μm.

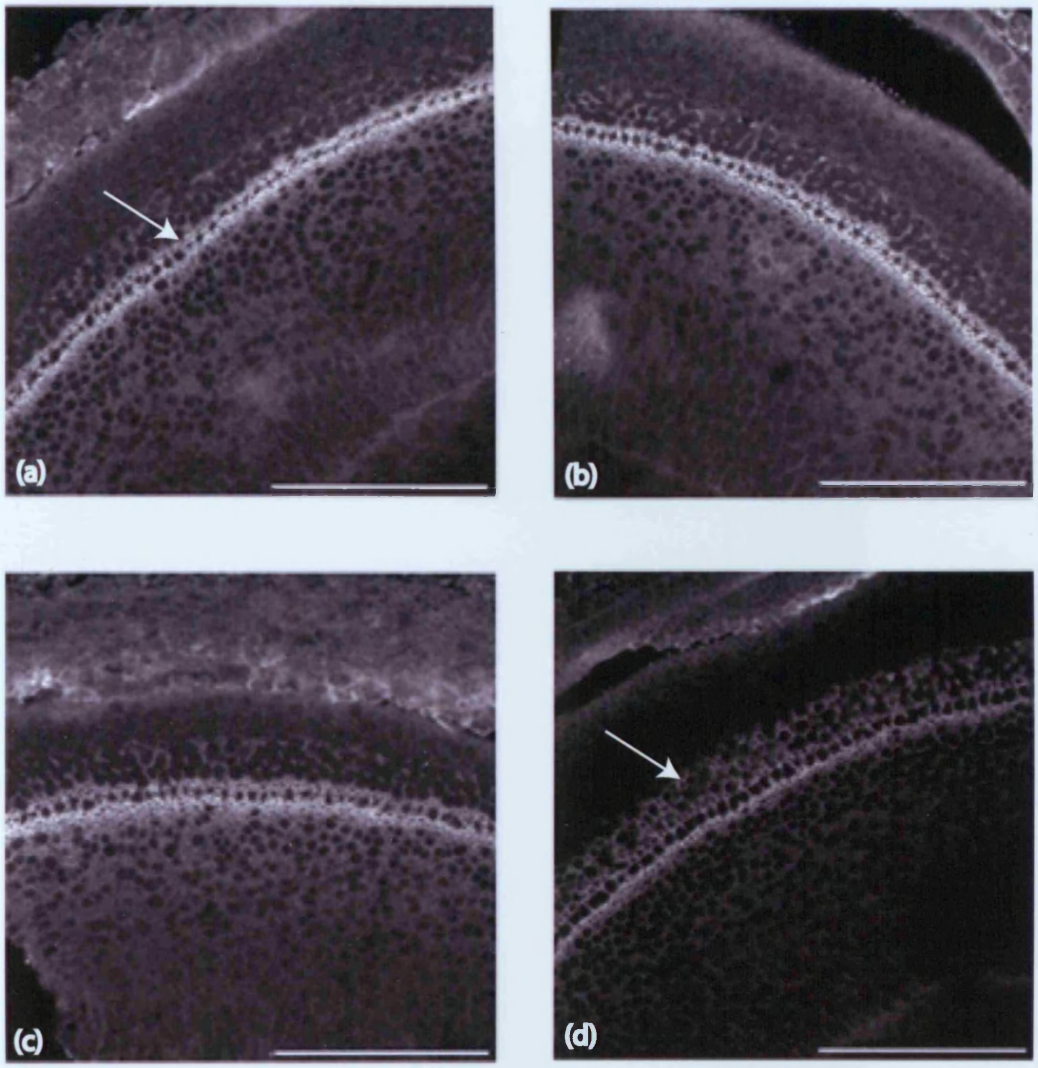


Figure 3.4

Jagged1 distribution in the basal cochlea at E15.5

In the more basal part of the cochlea at E15.5, the domain of Jagged1 staining appears to broaden as hair cells are produced.
The stain is still most intense in the region of the inner hair cells (arrow in a) , but staining becomes upregulated in a region outside these cells, in the supporting cells surrounding the outer hair cells. (arrow in d).
(Images a-d becoming more basal).
Scale bars are 100μm.

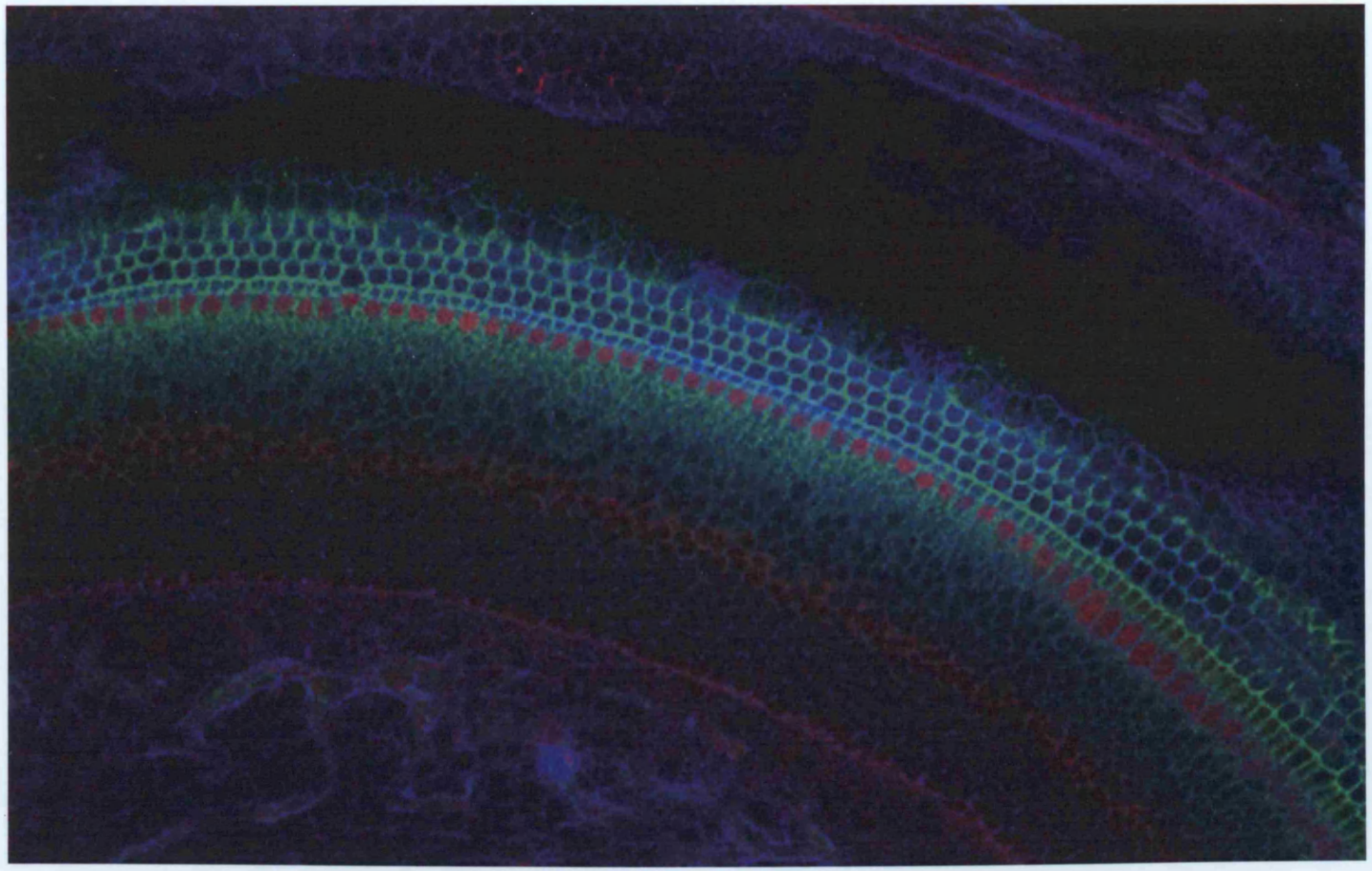


Figure 3.5

Jagged1 staining in the cochlea at E17.5

Jagged1 antibody (green) can be seen in the apical processes of supporting cells which separate hair cells from one another in the organ of Corti. A calretinin antibody (red) stains inner hair cells in this mid-basal region of the cochlea at E17.5. (Phalloidin staining of actin in blue.)

directly in contact with hair cells; the Deiters' cells, inner and outer pillar cells, and the inner phalangeal cells. Expression is apparently lost from the hair cells as they differentiate, although this is difficult to establish clearly from the antibody staining pattern alone.

At the medial border of the sensory patch, the level of Jagged1 protein decreases as one moves medially from the row of inner hair cells: a gradient of expression exists, going from a high concentration of Jagged1 protein in the membranes of supporting cells in contact with hair cells, and lower amounts in the numerous border cells that lie medially.

The pattern of Jagged1 varies along the length of the cochlea at E17.5, as it does at E15.5. In the apex, where hair cells have not yet begun to differentiate, Jagged1 protein is detected in a narrow band of cells similar to that seen at earlier embryonic stages. More basally, the pattern of Jagged1 broadens, and surrounds the developing hair cells that can be seen along the mid-apical to basal length of the cochlea.

3.2.4 Comparison of the patterns of Jagged1 and $p27^{Kip1}$ antibody staining in the cochlea

Both Jagged1 and $p27^{Kip1}$ have been reported to mark cells of the immature sensory patch. However, it has also been reported that the zone of Jagged1 positive cells does not exactly match that of $p27^{Kip1}$ in the developing sensory patch in the cochlea. Based upon immunostaining of adjacent sections of the cochlea, Chen et al have suggested that staining with the Jagged1 antibody does not overlap exactly with that of $p27^{Kip1}$. They report that it is expressed in a narrower band of cells that overlap with the patch of $p27^{Kip1}$ positive cells at its medial border, and that this is the site of hair cell production.

My own antibody staining on wholemount cochleas at E14.5 supports this finding. At this stage both antibodies stained a band of cells in the area of the prospective sensory patch that extended along its apicobasal axis. The two proteins differ in their pattern of distribution (Figure 3.6). As I described above, the band of staining produced with the Jagged1 antibody had a sharp lateral border and a diffuse medial border. This pattern of staining differs in several ways from that seen for $p27^{Kip1}$. The band of $p27^{Kip1}$ positive cells is much broader than the band of Jagged1 cells. In addition to this, the pattern of $p27^{Kip1}$ mirrors that of Jagged1, in that it has a sharp medial border of expression, with a diffuse

lateral border. Again, there is a decreasing gradient of signal at this diffuse border, with signal decreasing in more lateral cells.

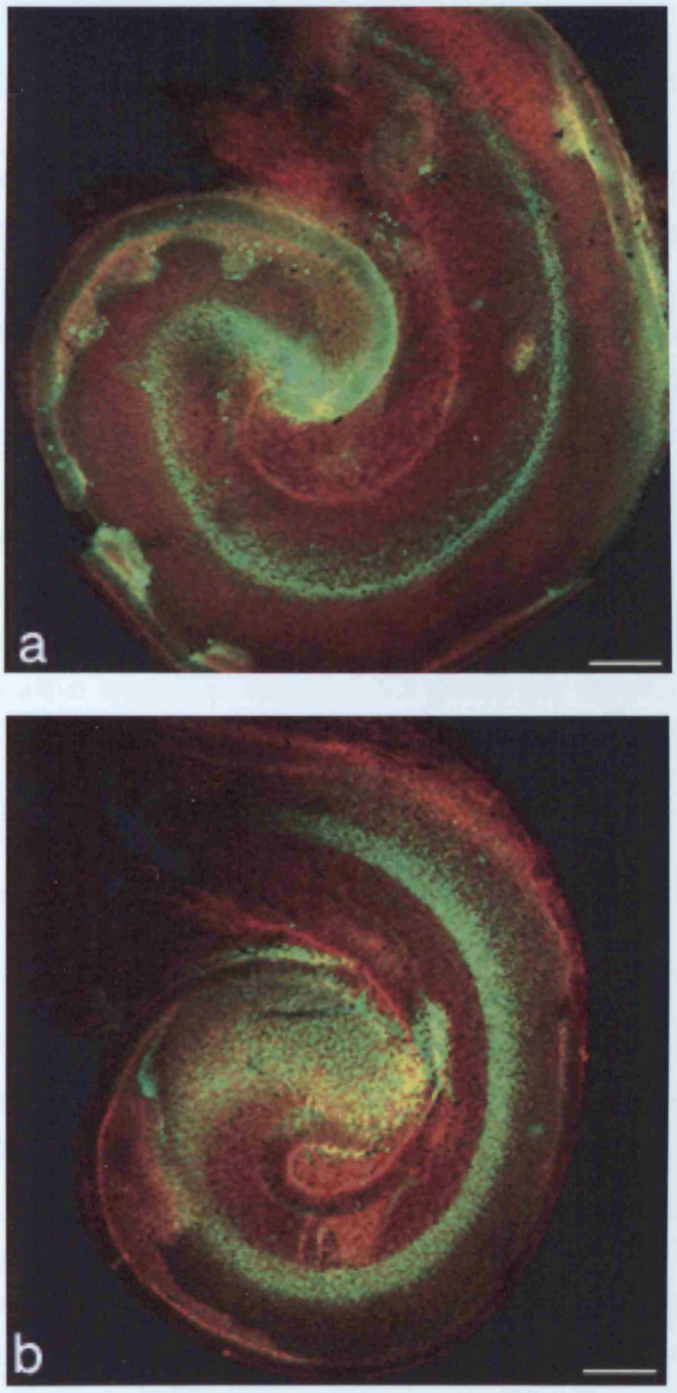


Figure 3.6
Comparison of Jagged1 and p27Kip1 in the cochlea at E14.5.
(a) Jagged1 antibody (green) stains a narrow band of cells that extends along the length of the cochlea.
(b)p27Kip1 antibody (green) marks a broader domain.
Scale bars are 100m.

Discussion

3.3.1 Jagged1 protein is not detected in all sensory patches early in ear development

Expression of Jagged1 in the otocyst at E10.5 occurs in a patch of cells in the middle third of the anteroventral wall. This finding is consistent with the published pattern of Jagged1 expression detected using in situ hybridisation. This is the area of the otocyst where *Lfng* is expressed at this time. It does not match the expression of *Bmp4*, which is seen in the posterior otocyst, where it is thought to mark the prospective cristae (Morsli, Choo et al. 1998). By E11.5, the patch of Jagged1 staining in the anteroventral area of the otocyst has divided into two separate domains. Again, this pattern of expression is similar to that of *Lfng*. No expression was detected in the posterior part of the otocyst. The early pattern of Jagged1 does not therefore seem to mark all future patches, although it is possible that Jagged1 is expressed in them at differing levels at these stages, and that the antibody staining is not sensitive enough to detect low but functionally significant levels of protein. In situ hybridisation reveals expression of Jagged1 in all of the sensory patches by E12.5, however (Morrison, Hodgetts et al. 1999).

3.3.2 The narrow domain of Jagged1 protein broadens as hair cells differentiate in the cochlea

At E14.5, when the majority of hair cells have undergone their terminal mitosis but are yet to start differentiating, Jagged1 is seen in a narrow band of cells along the length of the cochlea. Hair cell differentiation begins at E15.5, and hair cells can be identified in the basal part of the cochlea by their upregulation of actin. In the apical part of the cochlea at E15.5, Jagged1 protein distribution resembles that seen a day earlier. More basally, Jagged1 becomes upregulated and appears to surround the developing inner hair cells. At the base of the cochlea, the antibody stain brightly stains the cells surrounding the inner hair cells, and lower levels of signal can be detected in the cells surrounding the differentiating outer hair cells. In the hair cells themselves, *Jagged1* expression is ultimately lost. This has been clearly demonstrated by in situ hybridisation studies, and is supported by the immunofluorescence evidence from images such as that shown in Figure 5.12. The expression data suggest that the domain of Jagged1 broadens as differentiation proceeds, giving the impression that Jagged1 is perhaps switched on in cells of the

sensory patch later in development. However, this changing pattern of Jagged1 protein distribution may be an effect of cell rearrangements. The morphology of the patch changes over time, going from a thick epithelium, with nuclei at many different levels, to a bi-layered epithelium as seen in the mature patch. Jagged1 could then be said to be expressed in all cells of the sensory patch, which are then rearranged during development, making it appear as though the pattern of expression changes.

3.3.3 The distribution of Jagged1 and $p27^{Kip1}$ proteins are not identical

Both Jagged1 and $p27^{Kip1}$ have been suggested to mark the prospective sensory patch in the cochlea. However, the patterns of their protein distribution are not identical, suggesting that they mark different populations of cells.

$p27^{Kip1}$ appears to mark a zone of non-proliferting cells (ZNPC), thought to correspond to the sensory patch cells as they exit the cell cycle in synchrony between E12.5 and E13.5. Evidence that this may be the case comes from work done by Chen et al (2002), who compared expression of p27Kip1 with that of an early marker of hair cells, Math1. Expression of *Math1* was studied using $Math1^{GFP/+}$ mice, immunostained with an anti-GFP antibody. Math1 was first detected in a column of cells in the basal cochlea at E14.5, apparently immature hair cells at the medial border of the zone of the ZNPC. This column appears to span the apicobasal depth of the epithelium. Later in development, at E15.5, four rows of hair cells are seen located apically in the epithelium within the ZNPC.

Chen et al suggest that *Jagged1* expression overlaps with that of $p27^{Kip1}$ only at the medial border of the ZNPC, in the region that gives rise to hair cells. Based upon the detection of $Math1^{GFP}$, it seems possible that all hair cells are produced within this narrow domain. As mentioned earlier, there is evidence that *Jagged1* expression is positively regulated by Notch signalling. Furthermore, immature hair cells express other Notch ligands (Delta1 and Jagged2) strongly. It is possible, therefore, that as they migrate to their final positions in the epithelium, they deliver Notch signalling to cells they come into contact with in the ZNPC, causing these cells to activate expression of *Jagged1*, and to differentiate as hair cells. This interpretation is in agreement with the pattern of Jagged1 antibody staining I have observed in the cochlea as hair cells develop. It is not, however, in agreement with the idea that Jagged1 specifies sensory patch identity to all cells in the patch before hair cells differentiate. Rather, the pattern of Jagged1 antibody staining suggests that it may

mark cells that will form the patch from a pool of potential candidates marked by $p27^{Kip1}$. However, there could be an early low-level expression of Jagged1 in the outlying part of the prospective sensory patch - too little to be detected with the antibody but enough to function.

As I shall show in the next chapter, loss of Jagged1 has drastic effects on hair cell production in the outer hair cell region. The expression data for Jagged1 leave open the possibilities that this could reflect either an early function of Jagged1 within this region, or a function of Jagged1 at a relatively late stage through spreading of the Jagged1 expression domain from an initial early stripe that seems to correspond to the future inner hair cell region.

Chapter 4

The role of Jagged1 in Development of the Inner Ear

4.1 Introduction

There are three Notch ligands known to be expressed in the developing sensory epithelium of the inner ear: *Delta1* (*Dll1*), *Jagged2* (*Jag2*) and *Jagged1* (*Jag1*). Two of them, *Delta1* and *Jagged2*, are up-regulated in developing hair cells (Lanford, Lan et al. 1999; Morrison, Hodgetts et al. 1999). This pattern of expression is consistent with the idea that they mediate lateral inhibition and that their expression is negatively regulated by Notch signalling. *Jagged1*, however, has the opposite pattern of expression. It is expressed throughout the sensory patches early in their development, later becoming excluded from hair cells. As discussed previously, this Notch ligand does not appear to mediate lateral inhibition in the classical way. Notch signalling appears to positively regulate the expression of this ligand (Eddison, Le Roux et al, 2000).

The information concerning the role of *Jagged1* in the developing ear published to date suggests that *Jagged1* plays two major roles in development of the sensory patch, at different stages of its development. Early in development, *Jagged1* is expressed in the prospective patch, several days before hair cell production occurs, where it perhaps is required to specify the pro-sensory cells (Morrison, Hodgetts et al. 1999). This is supported by the analysis of *Jagged1* heterozygous mutant mice, in which some of the sensory patches are missing. Later in development, *Jagged1* is expressed in the supporting cells of the sensory patch, where an obvious suggestion would be that it is required for controlling the production of hair cells, preventing excessive numbers of cells becoming committed to this fate. Evidence that this might be the case comes from in vitro experiments, in which cochlea explants, which already contain the normal arrangement of hair cells and supporting cells, were treated with anti-*Jagged1* oligonucleotides. This resulted in the production of supernumerary hair cells, with two rows of inner hair cells, and multiple extra rows of outer hair cells (Zine, Van de Water et al. 2000). There are, however, important uncertainties about both suggestions. As an alternative to the first

suggestion, it has been proposed that the early expression of *Jagged1* may reflect a function in controlling the timing of differentiation, preventing hair cells from differentiating prematurely. It is not clear whether *Jagged1* expression at later stages affects proliferation, prosensory specification, or hair cell commitment.

In this chapter I describe experiments using conditional knockout mice to investigate the effect of homozygous loss of *Jagged1* upon development of the inner ear. Mice carrying a conditional allele of *Jagged1*, *Jagged1*^{flox}, have been crossed with mice expressing Cre recombinase under the control of the Foxg1 promoter, *Foxg1-Cre*.

4.1.1 Design and production of *Jagged1*^{flox} transgenic mice

The problem of early lethality seen in many mutant mice can be overcome by removing gene function only in restricted tissues. This can be achieved by using a site-specific DNA recombinase such as Cre. Cre recombinase originates from the bacteriophage P1, where it catalyses recombination events between 34bp recognition sequences called LoxP (location of cross-over in P1) sites. The basic Cre/LoxP system also works in mammalian cells, and is used to mediate recombination events in target genes. Regions of a gene can be removed by inserting LoxP sites into introns flanking the target sequence. Recombination of the LoxP sites can then be produced in a spatially and temporally restricted pattern by controlling Cre recombinase expression

Transgenic mice carrying a conditional allele of *Jagged1*, called *Jagged1*^{flox}, were produced by Katsuto Hozumi and Michael Owen (unpublished). In brief, the strategy for producing these mice was as follows. A targeting vector was constructed in which a neomycin selection cassette, composed of the neomycin coding sequence flanked by LoxP sites, was inserted into the intron upstream of exon 4 of *Jagged1*. A third LoxP site was inserted downstream from exon 5 (Figure 4.1). This targeting vector was electroporated into ES cells, and homologous recombinant clones were selected on the basis of their neomycin resistance. The selected clones were then transiently transfected with a plasmid expressing Cre recombinase. Clones in which a partial recombination had occurred, where the neomycin resistance cassette had been removed leaving two LoxP sites flanking exons 4 and 5, were identified using Southern blot analysis. Several such clones were used in blastocyst injections, and founder mice were selected on the basis of

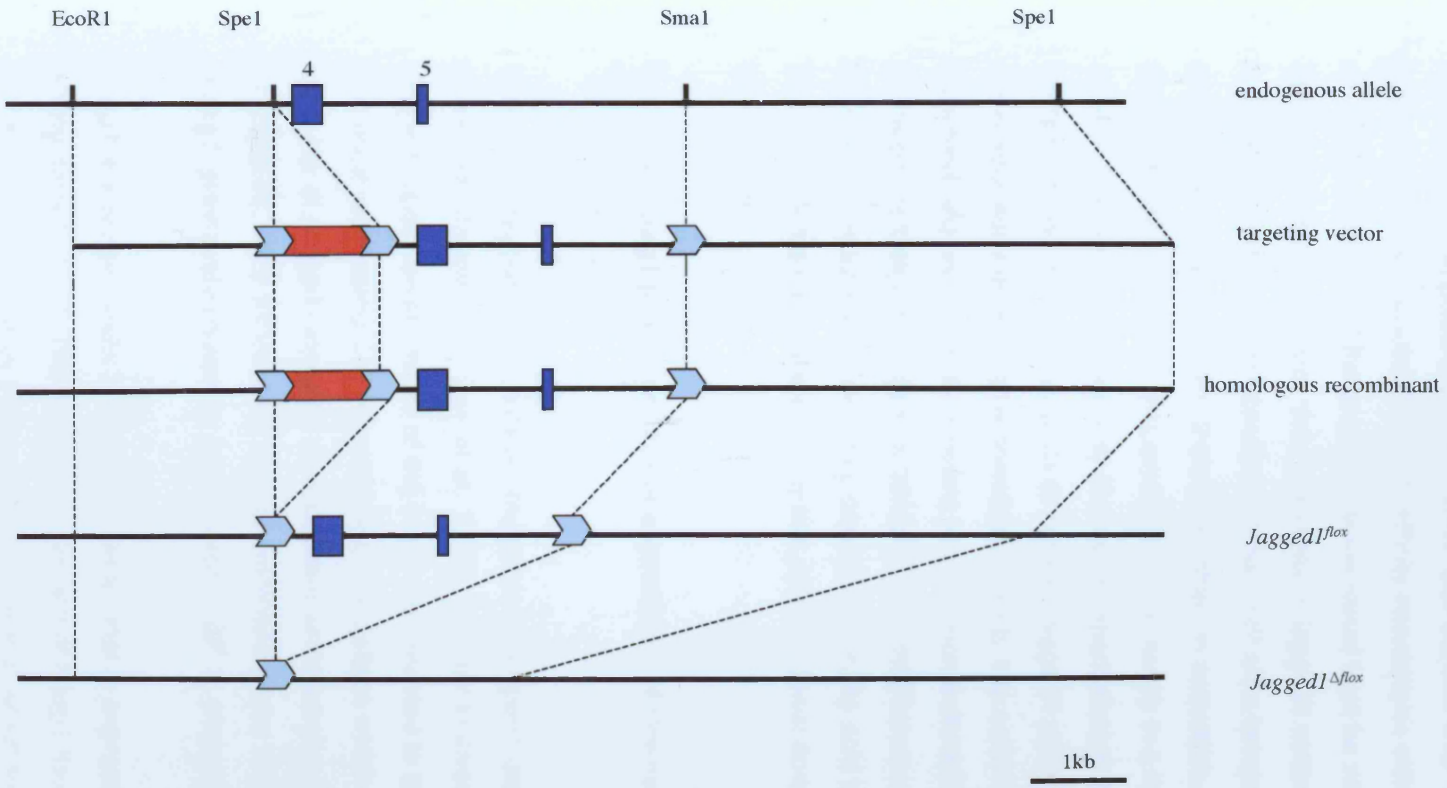


Figure 4.1

Schematic showing the strategy for producing *Jagged1* conditional allele.
Exons 4 and 5 (dark blue boxes) were flanked by LoxP sites (light blue arrows) and a Neomycin resistance cassette was inserted upstream of the targeted sequence (red box). Partial recombination of the allele was performed in vitro to remove NeoR and transgenic mice were produced with the 2-Lox construct. Recombination of the LoxP sites removes exons 4 and 5 of the *Jagged1* gene, and introduces a frameshift causing translation to terminate in a stop codon in exon 6.

southern blotting and PCR to reveal the presence and the transmission of the *Jagged1*^{flox} allele.

Exons 4 and 5 encode the DSL domain and the first EGF-repeat of the Jagged1 protein, both of which are required for high affinity interactions with Notch receptors (Shimizu *et al*, 1999). Removal of these exons alone would thus be sufficient to produce a loss of function allele. In addition, the downstream *Jagged1* coding sequence is disrupted in the recombined allele. Recombination of the LoxP sites brings exons 2 and 6 into consecutive order. Exons 1, 2 and 6 are then transcribed in sequence. The translational frame-shift caused by this abnormal sequence of exons results in a novel stop-codon shortly after exon 2. The protein encoded by the recombined allele is therefore severely truncated, containing only 147 amino acids of normal Jagged1 protein followed by 26 amino acids of novel sequence produced as translation reads into exon 6. To determine whether this truncated Jagged1 protein contains any functional domains, the sequence was submitted to the online bioinformatics tool SMART (Simple Modular Architecture Research Tool) from EMBL. This programme identifies conserved amino acid sequences and predicts possible functional domains on the basis of this. No functional domains were found using this programme.

4.1.2 The *Foxg1* promoter drives expression of Cre-recombinase in the developing ear

Mice homozygous for a knockout mutation of *Jagged1* die at E10.5 as a result of defects of the vasculature (Xue, Gao et al. 1999). In order to investigate the effect of loss of *Jagged1* upon development of the inner ear I wanted to cross mice carrying *Jagged1*^{flox} with mice expressing Cre recombinase in a pattern which would recombine the allele in the inner ear at an early stage of development while leaving it intact in the developing vasculature. Mice expressing Cre recombinase under the control of the *Forkhead box G1* (*Foxg1*, previously known as *brain factor-1*, *BF-1*) promoter meet these requirements.

Foxg1 is a winged-helix transcription factor that is expressed in several different tissues during development. The expression pattern of *Foxg1* has been investigated using both in situ hybridisation and by using a strain of mice in which the coding sequence of the *Foxg1* gene has been replaced with the *LacZ* gene (Xuan, Baptista et al. 1995). I will describe the expression pattern of *Foxg1* as detected using both methods.

Expression of *Foxg1* in normal mice is first detected at the 6-somite stage (around E8), as the neural folds begin to form, in the anterior neural ridge (Figure 4.2). Scattered cells are later seen in the anterior and lateral surface ectoderm. As development proceeds, this zone of expression extends caudally to the area of the midbrain and the rostral part of the hindbrain. Expression is also seen in the anterior part of the developing foregut at this stage. At E9.5, *Foxg1* expression is seen in a restricted pattern in the telencephalic vesicles, the foregut, the nasal half of the optic vesicle, the otic and olfactory placodes, the pharyngeal pouches, and the intermediate and epibranchial placodes (Hatini, Ye et al. 1999; Hébert and McConnell 2000).

Foxg1-Cre mice were produced by modification of an existing targeting construct previously used to make transgenic mice, in which the *β-Galactosidase (β-Gal)* coding sequence was inserted, in-frame, after the first 13 amino acids of the *Foxg1* gene (Xuan, Baptista et al. 1995). The *β-Gal* coding sequence was subsequently replaced with that of Cre recombinase, with an SV40 intron and a poly(A) sequence (Hébert and McConnell 2000). The *Foxg1-Cre* allele is thus a knockout allele of *Foxg1*. Homozygous *Foxg1* knockouts die at birth with breathing difficulties. They exhibit a severe reduction in the size of the cerebral hemispheres resulting from decreased proliferation and premature neuronal differentiation (Xuan, Baptista et al. 1995). Heterozygous *Foxg1* knockout mice appear normal and viable and the colony is maintained as heterozygotes. It is nevertheless possible that the heterozygous loss of *Foxg1* could contribute to the phenotype seen in *Jagged1* conditional knockout mice, so in my experiments I used a *Foxg1-Cre* heterozygote as a littermate control where possible.

The *Foxg1-Cre* mice have been thoroughly characterised and have been used to recombine conditional alleles in the developing ear successfully (Hébert and McConnell 2000; Pirvola, Ylikoski et al. 2002). Part of this characterisation was to determine the pattern of expression and activity of Cre when it is under the control of the *Foxg1* promoter. Radioactive in situ hybridisation detected Cre RNA in a pattern almost identical to that seen for the normal *Foxg1* gene at mid-embryonic stages. The pattern of Cre activity was then analysed using two lines of reporter mice, ROSA26 Reporter (R26R) mice and Z/AP mice, in which Cre mediated recombination switches on expression of LacZ or human placental alkaline phosphatase respectively. *Foxg1* successfully recombined both conditional alleles in vivo (Hébert and McConnell 2000).

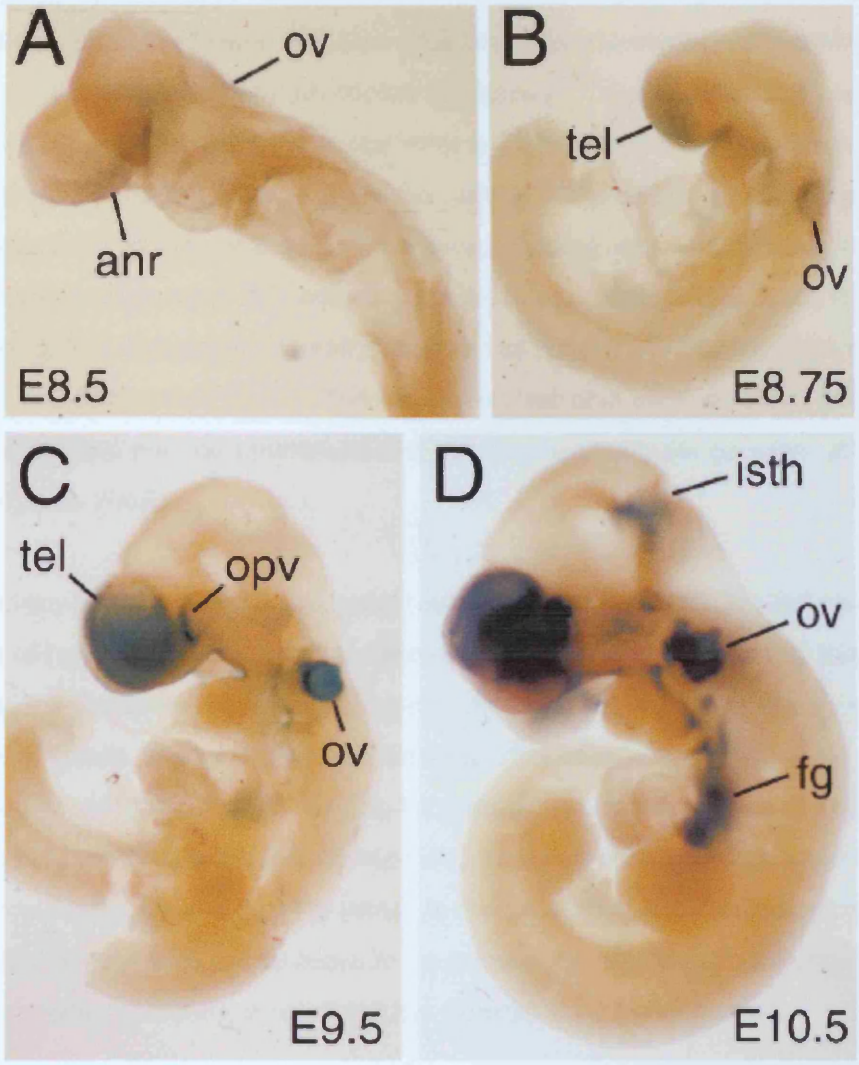


Figure 4.2

Z/AP reporter activity after tissue specific recombination of the conditional allele by Foxg1-Cre.
A. At E8.5 recombination is detected in the otic placode (marked as the otic vesicle, OV)) and in the anterior neural ridge (ANR).
B. At E8.75, recombination is detected in the telencephalon (tel) and also in the otic cup.
C. At E9.5 recombination is also seen in the optic vesicle (OPV).
D. At E10.5 recombination is detected in the isthmus (isth) and in the foregut (fg).
(Reproduced from Hebert and McConnell, 2000)

For my experiments, the pattern of expression of *Foxg1-Cre* in the developing otocyst is of primary importance. Expression needs to be switched on early in ear development, and to occur throughout the tissue. It is also desirable that expression should persist in this tissue to ensure recombination of both copies of *Jagged1*^{flox} in all cells. The *Foxg1* promoter does indeed drive expression of Cre in the inner ear early in its development. At E8.5 *Foxg1-Cre* recombined the R26R allele in scattered cells of the otic placode. Sections of the otocyst from Foxg1-Cre:R26R mice at E11.5 showed staining with X-Gal throughout. Radioactive in situ hybridisation on E12.5 embryos showed expression of Cre in the developing ear, suggesting that expression is maintained in this tissue over several days of development (Hébert and McConnell 2000). Recombination has also been detected in Foxg1-Cre:Z/AP mice throughout the otic epithelium and the cochleovestibular ganglion at E13.5 (Pirvola, Ylikoski et al. 2002).

The pattern of recombination detected using these reporter strains did not match the pattern of normal *Foxg1* expression exactly. In addition to those areas that normally express the gene, recombination was detected in the midbrain-hindbrain boundary, and in the lens placode. These additional restricted sites of expression were not of concern when considering using the *Foxg1-Cre* line for producing conditional loss of *Jagged1,* as the pattern of recombination is still restricted to tissues where the gene is not required for embryo survival. A more serious threat to viability is that the Cre recombinase expression under the *Foxg1* promoter appears to be more generally "leaky", with recombination of the reporter alleles occurring throughout the embryo in a minority cases.

The extent of ectopic recombination seen differed with different genetic backgrounds. Hébert and McConnell crossed *Foxg1-Cre* mice onto several different genetic strains, and analysed the pattern of recombination of a reporter allele resulting in each case. While ectopic recombination was seen on all backgrounds studied, the most resricted pattern of recombination was seen on a 129SvJ background. These mice were unavailable, so experiments were started on mice on a C57BL/6J background, on which the majority of mice showed little ectopic recombination of the reporter allele (Hébert and McConnell, 2000). The 129SvJ strain was acquired, but because a decent proportion of conditional knockout mice survived to late embryonic stages on the C57BL/6J background, and because of time restraints, were not used.

4.2 Results

Here I describe the experiments I have performed investigating the role of *Jagged1* in the developing ear. I deal first with the validation of the transgenic mice carrying a conditional allele of *Jagged1*, *Jagged1*^{flox}. This work carried on from that done by Katsuto Hozumi in producing and maintaining the *Jagged1*^{flox} mice. I then go on to show that the strategy for producing conditional knockout mice, using *Foxg1-Cre* mice, has been successful. Having demonstrated this, I describe the inner ear defects seen in the *Jagged1* conditional knockout mice.

4.2.1 Validation of *Jagged1*^{flox} construct

This project began with two lines of *Jagged1*^{flox} mice, called 5C5-8 and 5G3-4, given to me by Katsuto Hozumi. These mice were maintained on a C57BL/6J background, and mice were genotyped by PCR using primers designed by K.Hozumi that flank the 5' LoxP site. Mice from the 5C5-8 colony were crossed with *Foxg1-Cre* mice to generate conditional knockout mice.

A technical difficulty was encountered with the *Jagged1*^{flox} mice early in the project. Experiments conducted by Katsuto Hozumi, who was using them for studies of the immune system suggested that recombination of the allele was not occurring in vivo. One possibility for this was that the LoxP sites were not intact. Routine genotyping of both lines was done using primers that flanked the 5' LoxP site, so this site was clearly present in both 5C5-8 and 5G3-4 strains of mice (details of primers are given in Materials and Methods). To determine whether the 3' LoxP site was present in both cases, I designed PCR primers that flanked the site of its insertion in the intronic sequence downstream from exon 5. PCR on genomic DNA isolated from members of both strains revealed that while the strain I had been using (5C5-8) lacked the 3' LoxP site, the second strain (5G3-4) carried the complete *Jagged1*^{flox} insert.

Before proceeding with crossing the 5G3-4 line with *Foxg1-Cre* mice, I wanted to check that the LoxP sites were of the correct sequence in the 5G3-4 strain. Sequencing was performed as described in Chapter 2. The normal 34bp LoxP sequence is composed of inverted 14bp repeats, the recombinase binding elements (RBEs), either side of an 8bp

core spacer region. I found that the inverted repeats and the core spacers of both LoxP sites had the expected sequences, and so would be predicted to interact with Cre recombinase molecules correctly. All subsequent experiments were conducted using the 5G3-4 line of Jagged1^{flox} mice.

4.2.2 Recombination of *Jagged1^{flox}* produces a knockout allele

Having established that both LoxP sites were intact, and that they were inserted in the correct intronic sequence of the *Jagged1* gene, I wanted to determine whether the *Jagged1^{flox}* allele was recombined in vivo by *Foxg1-Cre*, and whether the recombined *Jagged1^{flox}* allele would result in a loss of function of *Jagged1*. To test this, I analysed litters from matings between mice with the genotypes *Foxg1$^{Cre/+}$:Jagged1$^{flox/+}$* and *Foxg$^{+/+}$:Jagged1$^{flox/+}$*. I then genotyped each embryo as described in Materials and Methods.

I used a PCR primer (designed by Katsuto Hozumi) that matched sequence downstream from the 3' LoxP site (3Jspe) (Figure4.3). When used with a primer upstream from the 5' LoxP site (23JSma), a small PCR product is produced only from the recombined allele under the restrictive PCR conditions I used. In the intact *Jagged1^{flox}* locus, the primers flank over 4kb of genomic sequence, which is not amplified in the PCR conditions I used. Genomic DNA isolated from tail biopsies from the embryos was used to determine whether the *Jagged1^{flox}* allele was recombined in those mice with *Foxg1-Cre*. Recombination of the allele was detected in all cases where a *Jagged1^{flox}* allele was carried in the presence of *Foxg1-Cre*.

As described above, the recombined allele is predicted to produce a truncated messenger RNA due to a stop codon in non-sense sequence following the splicing of exon 2 to exon 6. To check that the predicted transcript is produced from the recombined allele in vivo, RT-PCR was performed on conditional knockout mice. Primers targeted against sequence of exon 2 and exon 6, which lie either side of the site of recombination, produced a PCR product of the expected size using total RNA as a template (Figure 4.3). Thus, the conditional allele is recombined successfully by *Foxg1-Cre*, in the intended fashion.

In parallel experiments, I addressed the question of whether the recombined allele was indeed functionally null, and whether the phenotype shown by mice homozygous for the

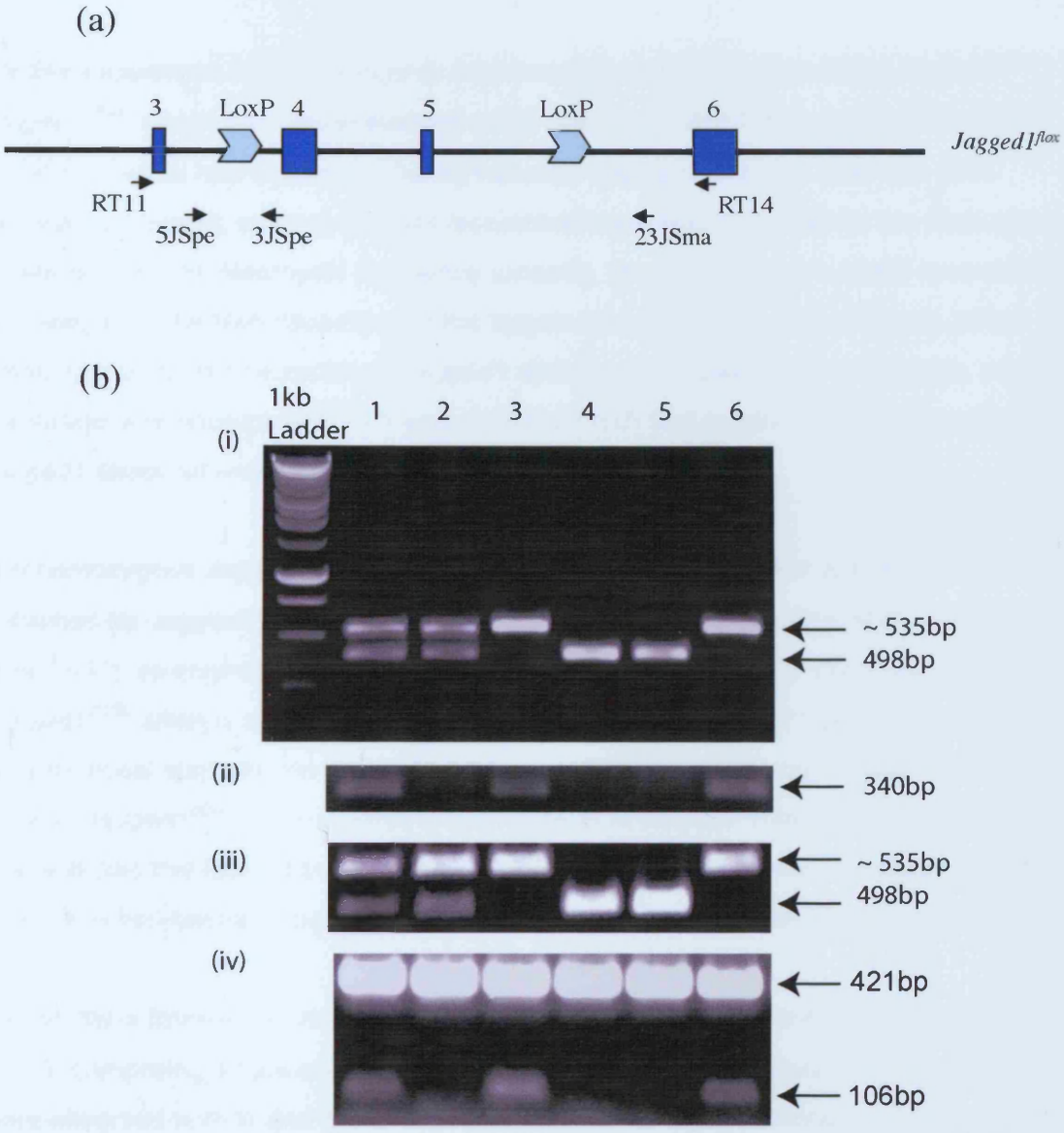


Figure 4.3

Genotyping of *Jagged1* conditional knockout litters, and detection of the recombined conditional allele by RT-PCR.
(a) Schematic showing the location of primer binding in the *Jagged1* conditional allele.
(b) Genotyping PCR and RT-PCR results for one conditional knockout litter (Lanes 1-6 correspond to embryos 1-6 of the litter).
Gel (i) shows PCR results using 5JSpe and 3JSpe. Two bands are produced in heterozygous *Jagged1flox* individuals (e.g. Embryos No1) and a single band is seen in homozygous *Jagged1flox* mice (No3 and No6) and in the wildtype mice (No4 and 5). Gel (ii) shows Cre PCR results. No3 and No6 are thus homozygous *Jagged1* conditional knockout mice. Gels (iii) and (iv) show RT-PCR results on the same litter. The same primers are used in (iii) as were used in the case of gel (i), as a quality control. Gel (iv) shows the wildtype gene product (band size 421) using primers RT11 and RT14). All mice carrying both the conditional allele and *Foxg1-Cre* have a smaller band, size 106bp) indicating that the conditional allele has been recombined, as expected.

recombined allele in my mice was the same as that published for other *Jagged1* knockout mice.

For this experiment I used transgenic mice carrying a copy of recombined *Jagged1*^{flox}, *Jagged1*$^{\Delta flox}$, which were generated using ES cells in which a full recombination of the conditional allele had occurred. (During transient transfection of ES cells with Cre-expressing plasmid, several different recombination events occur. While the main aim was to remove only the Neomycin resistance cassette, some alleles were totally recombined, removing both the Neo cassette and the targeted portion of the *Jagged1* gene.) Mice heterozygous for the recombined *Jagged1* allele were crossed in timed matings, and the homozygous phenotype at E10.5 was compared with that published for homozygous *Jagged1* knockout mice.

The homozygous *Jagged1*$^{\Delta flox}$ mice did indeed have defects similar to those previously published for *Jagged1* homozygous knockout mutant mice (Xue, Gao et al. 1999 and Tsai et al, 2001), as originally described for homozygous *Jagged1*^{dDSL} individuals. The *Jagged1*^{dDSL} allele is a deletion of 5kb at the 5' end of the *Jagged1* gene including the transcriptional start site: no *Jagged1* RNA or protein can be detected in the *Jagged1*^{dDSL} mutant. *Jagged1*^{dDSL} homozygotes exhibit defects in vascular remodelling, manifest as a pale yolk sac that lacks the normal pattern of blood vessels. The homozygous embryos often exhibited haemorrhaging, and an enlarged pericardium was also been observed.

Eleven litters from timed matings of *Jagged1*$^{\Delta flox}$ heterozygous parents were collected at E10.5, comprising a total of 81 mice, of which 14 were homozygous mutants. Embryos were observed both in and out of their yolk sacs under the dissecting microscope. As in homozygous *Jagged1*^{dDSL} mice, most homozygous *Jagged1*$^{\Delta flox}$ embryos at E10.5 (9/14) had pale yolk sacs (Figure 4.4), in which normal-sized blood vessels could not be seen. Of the remaining five individuals, one had a well-established vascular network in the yolk sac. Large blood vessels could be seen in the other four homozygotes, though they did not appear to be associated with smaller vessels. While there was some variation in the severity of the disruption of blood vessel patterning in the yolk sac, these observations are largely in agreement with expectations if *Jagged1*$^{\Delta flox}$ is a null mutation.

Moreover, some of the homozygous embryos had an enlarged pericardium (5/14), as has been previously reported in *Jagged1* mutants. Most embryos were pale and bloodless, and

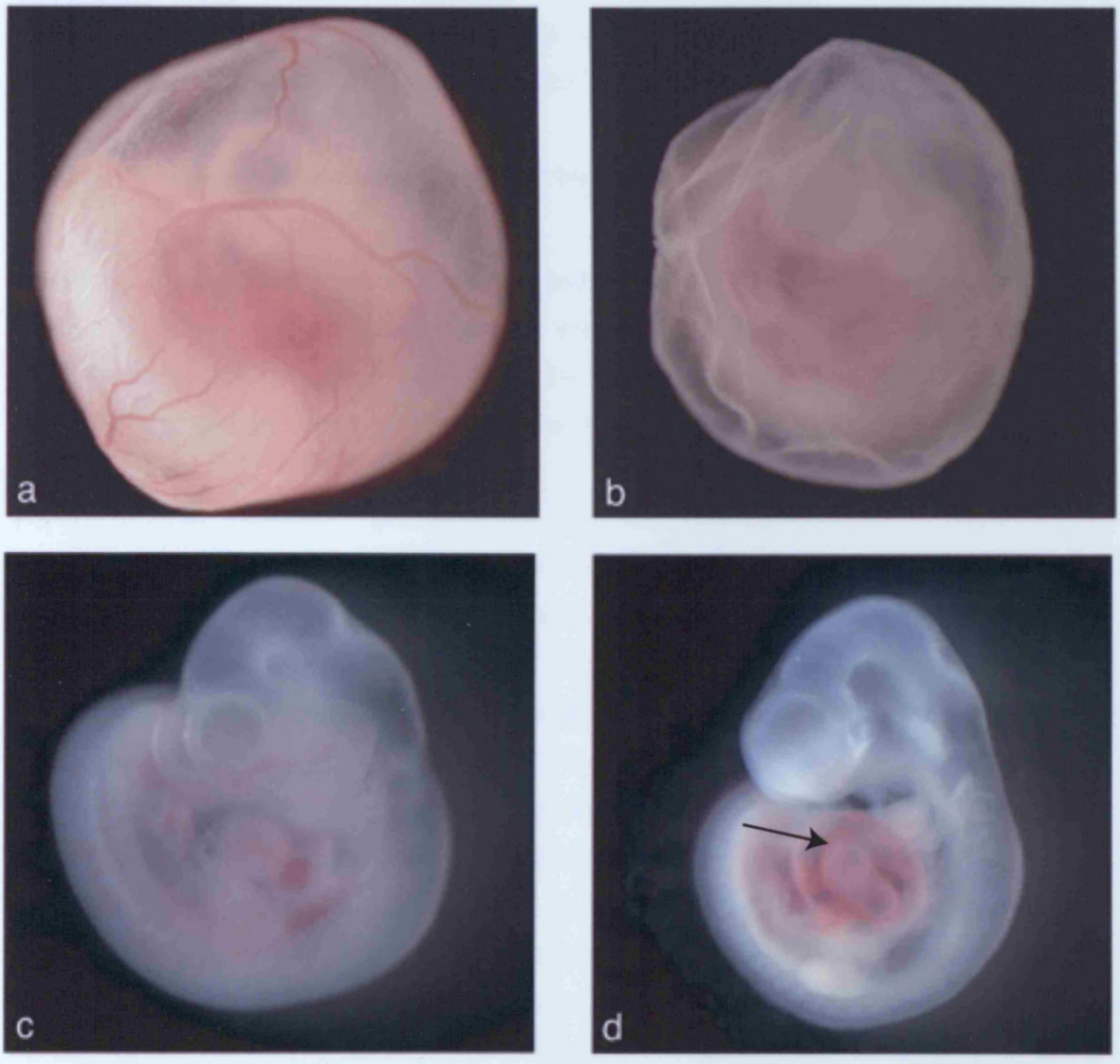


Figure 4.4

Mice that are homozygous for a germline recombination of the *Jagged1* conditional allele exhibit defects similar to those seen in other strains of *Jagged1* knockout mice. There is a loss of blood vessels in the yolk sac in the mutant mice (b compared to the wildtype situation in a), and embryos exhibited heamorrhaging and enlarged percardia (arrow in d), compared to littermate controls (c).

some exhibited haemorrhaging. In addition, most homozygous individuals were delayed in development compared to their littermates by 12-24 hours. The above phenotype is in agreement with the published *Jagged1* knockout phenotype, and supports the expectation that the recombined *Jagged1*^{flox} allele, *Jagged1*$^{\Delta flox}$, is a loss-of-function allele of *Jagged1*.

4.2.3 Early embryonic lethality is avoided by tissue specific removal of *Jagged1*

It was hoped that tissue specific loss of *Jagged1* would allow observation of the knockout phenotype in the inner ear at postnatal or at least late embryonic stages of development. At these stages, the majority of hair cells in the cochlea are in the process of differentiating, and can easily be identified on the basis of their morphology.

For this purpose, I wanted to generate mice that carried two copies of the *Jagged1*^{flox} allele, *Jagged1*$^{flox/flox}$, and one copy of *Foxg1-Cre* (*Foxg1*$^{Cre/+}$). In a mouse of this genotype both copies of *Jagged1* would be removed in the regions of *Foxg1-Cre* expression. I also required wildtype, (*Jagged1*$^{flox/flox}$: *Foxg1*$^{+/+}$) or (*Jagged1*$^{flox/+}$: *Foxg1*$^{+/+}$) or (*Jagged1*$^{+/+}$: *Foxg1*$^{Cre/+}$) embryos for use as control littermates. *Foxg1*$^{Cre/+}$ mice were used as controls where possible as loss of one copy of Fogx1 could itself have phenotypic consequences. Indeed, homozygous *Foxg1-Cre* mice die at birth with severe developmental defects, and exhibit defects in hair cell patterning in the cochlea. In order to get litters containing mice of these genotypes, stud male mice that were double heterozygotes for *Foxg1-Cre* and *Jagged1*^{flox} (*Jagged1*$^{flox/+}$: *Foxg1*$^{Cre/+}$) were crossed with *Jagged1*$^{flox/+}$ females in timed matings. The litters from parents with these genotypes are predicted to contain homozygous conditional knockout mice (*Jagged1*$^{flox/flox}$: *Foxg1*$^{Cre/+}$) at a frequency of one in eight.

In order to study hair cell production in the inner ear I needed to collect conditional knockout mice at early postnatal or late embryonic stages. 499 mice were collected and genotyped as described in Materials and Methods, between E16.5 and P0. The *Jagged1* conditional knockout mice were easily distinguished from their littermates, being markedly smaller, and having a striking malformation of the iris and haemorrhaging of cranial blood vessels (Figure 4.5). Of the expected 62 homozygous conditional knockout mice expected from this number of individuals (based upon an expected frequency of one in eight), only 37 were found. Thus, only 60% of conditional knockout mice survive to late

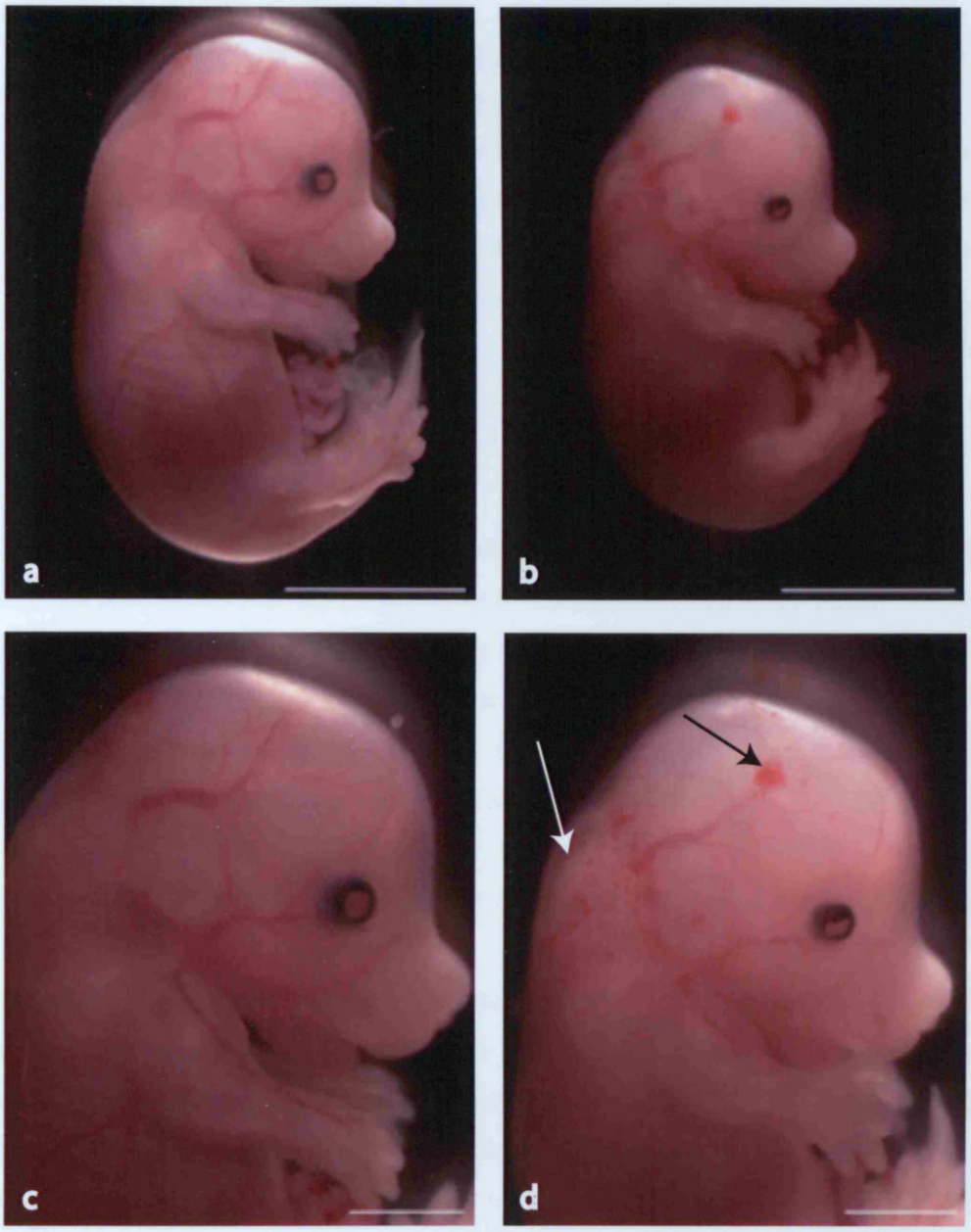


Figure 4.5

*Jagged1*conditional knockouts collected at E14.5 (b) are smaller than normal littermates (a). They exhibit haemorrhaging in the cranium, and oedema (arrows in (d), as well as a striking iris defect.

Scale bars are 5mm (a,b) and 3mm(c,d).

embryonic/early postnatal stages, meaning that of the 64 litters of mice collected, processed and genotyped, about half contained *Jagged1* conditional knockout mice.

To determine when the other 40% of *Jagged1* conditional knockout mice were dying, I examined litters from the matings of the type described above at different stages. When collected at early embryonic stages, between E9.5 and E11.5, 12 out of the 114 embryos collected were conditional knockouts. This figure is close to the predicted number of 14 conditional knockout mice in this number of progeny, based upon the expected frequency of one in eight. Of the 12 conditional knockout individuals, two were found dead and were haemorrhagic. Thus, at early embryonic stages most conditional knockout mice appeared healthy and lacked obvious defects in morphology and in the developing heart and blood vessels.

Of the litters collected a few days later in development, between E12.5 and E15.5, 20 conditional knockout mice were found in the 199 embryos collected. Again, this figure is close to the expected 25 conditional knockout mice, and more than the number (15) expected for a 60% suvival rate.. A large proportion of conditional knockout mice thus appear to die after completing the second week of gestation, although more litters would be needed to set this conclusion on a firm statistical basis.

4.2.4 *Jagged1*^{flox} is recombined early in the developing inner ear

My aim was to determine the effect of a total loss of *Jagged1* upon development of the inner ear. This requires that recombination of the *Jagged1*^{flox} allele occurs early in ear development, preferably before *Jagged1* expression is switched on, and that *Jagged1* is removed throughout the developing ear. The earliest expression of *Jagged1* in the developing mouse ear has been detected by in situ hybridisation at E9.5 in the ventral part of the otocyst (Mitsiadis, Henrique et al. 1997). Foxg1 driven Cre recombinase has been shown to be active in scattered cells of the otocyst a day earlier, at E8.5, using Z/AP reporter mice (Hébert and McConnell 2000).

To determine whether recombination of the *Jagged1*^{flox} allele occurs successfully at these early stages of ear development, I used a *Jagged1* antibody to stain sections of conditional knockout embryos. The *Jagged1* antibody was a commercially available, affinity-purified polyclonal rabbit antibody raised against a peptide that mimics part of the C-terminus

portion of the *Jagged1* protein (Santa Cruz, C-20). The truncated protein encoded by the recombined *Jagged1*^{flox} allele completely lacks the C-terminal, and thus would not be detected.

Litters containing conditional knockout mice were collected at early embryonic stages, fixed and embedded for cryosectioning. Sections through the otocyst were selected from conditional knockout mice and from wildtype or *Foxg1-Cre* heterozygote littermate controls, and were stained with the anti-Jagged1 antibody and fluorescent phalloidin. Confocal images were taken of every section through both the left and right otocysts of three conditional knockout individuals and two corresponding control embryos.

At E9.5, no *Jagged1* expression could be detected in the otocyst using this antibody in either control or conditional knockout embryos. By E10.5, a patch of Jagged1 positive cells is seen in the ventrolateral part of the otocyst in control mice (Figure 4.6), but no Jagged1 was detected in any part of the otocyst in any of the three conditional knockout mice. Recombination of the *Jagged1*^{flox} allele must thus have occurred by E10.5, perhaps much earlier, and must have occurred throughout the otocyst, or at least throughout the Jagged1 expression domain.

I also carried out a similar, though less exhaustive, analysis of the eye in the same mice. Foxg1-Cre recombines R26R in the lens and in the nasal half of the retina at E10.5 (Hébert and McConnell 2000). A loss of *Jagged1* would thus be expected to have occurred by E10.5. At this stage *Jagged1* positive cells are normally seen at the equatorial margins of the developing lens, and in the ciliary margins of the retina. This was indeed the case in the control littermates analysed here. No *Jagged1* was detected in the lens of any of the three conditional knockout mice. Staining was also lost in the ciliary margins of the retina except in one case, where some residual *Jagged1* was detected by the antibody in the ciliary margin of the retina of one conditional knockout individual. This staining was seen in the temporal half of the retina, where *Foxg1-Cre* is not expected to be expressed. No such low level staining was detected in the conditional knockout ears.

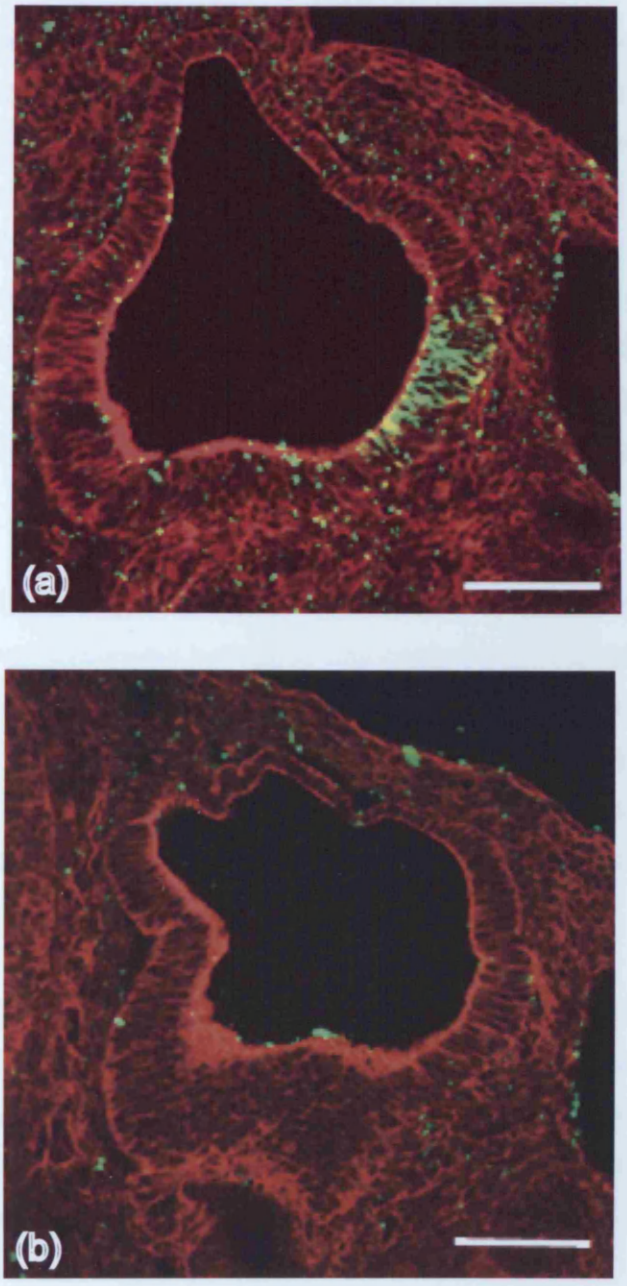


Figure 4.6
Jagged1 protein cannot be detected in the Jagged1 conditional knockout at E10.5.

Sections are oriented so that lateral is right, and dorsal is top. In the wildtype littermate, Jagged1 antibody staining (green) marks a patch of cell in the ventrolateral otocyst. No such staining can be seen in the *Jagged1* conditional knockout otocyst at this stage (b). Red stain is phalloidin.
Image (a) was seen previously in Figure 3.1.
Scale bars are 100μm.

4.2.5 Loss of *Jagged1* causes gross morphological defects of the inner ear

Jagged1 conditional knockout mice were easily identifiable upon collection at E17.5 and on dissection of the inner ear showed gross morphological defects. The inner ear from these mice appeared smaller than in their littermates, and had obviously abnormal semicircular canals. In order to study the defects of morphology in detail, inner ears dissected from E17.5 *Jagged1* conditional knockout individuals and their corresponding littermate controls were cleared in methyl salicylate and were injected with a solution of latex paint to reveal the endolymphatic space. The morphology of the inner ear was also studied using serial sections of the head from *Jagged1* conditional knockout mice and their littermate controls. stained with either fluorescent phalloidin or Haematoxylin and Eosin (H & E).

In all cases studied, the superior semicircular canal and its accompanying ampulla were lost in the *Jagged1* conditional knockout. The posterior semicircular canal was severely truncated, and the posterior ampulla was also missing. The horizontal semicircular canal was also truncated, but the horizontal ampulla was present, though reduced (Figure 4.7). In some cases the utricle was also missing, though the saccule was present in all cases studied. These defects of the semi-circular canals were already present at E13.5, when these structures have just formed (Figure 4.8).

The gross morphology of the cochlea was also affected by loss of *Jagged1*. The length of the cochleas from E17.5 individuals was measured using confocal images of flat-mounted pieces of cochlea (cut into apical, middle and basal portions) and Zeiss LSM 5 Image Browser software (as described in Materials and Methods) (Table 4.1). Cochleas from *Jagged1* conditional knockout mice were between 60% and 80% of those from control littermates.

4.2.6 Loss of *Jagged1* results in a loss of vestibular sensory patches

In order to study effects on vestibular patch development in more detail, I immunostained serial cryosections of *Jagged1* conditional knockouts and their littermate controls at E17.5 with fluorescent phalloidin, and either Jagged1 or calretinin antibodies. Calretinin is a calcium-binding protein that is present in the cytoplasm of post-mitotic hair cells, becoming detectable shortly after they have undergone their terminal mitosis (Zheng and Gao 1997).

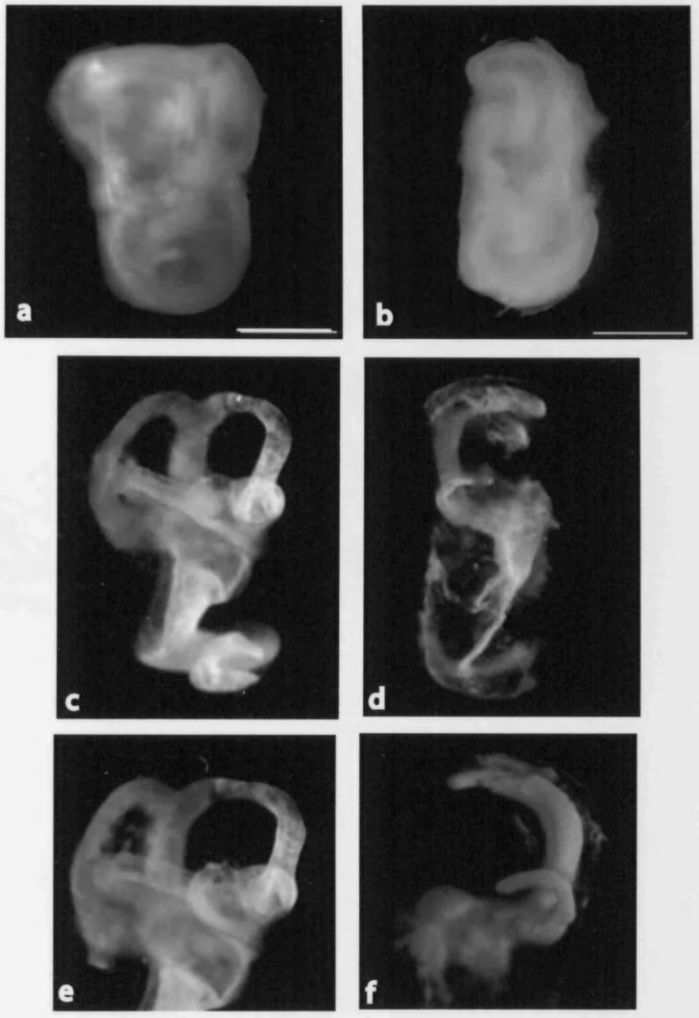


Figure 4.7

Morphological defects seen in the *Jagged1* conditional knockout at E17.5. Darkfield images of the inner ears dissected and fixed overnight reveal striking defects in the vastibular apparatus (control in a, and conditional knockout in b, medial view). Paintfilling of the inner ear shows this defect in more detail (lateral views shown). Wildtype inner ear is shown in (c), and a higher magnifcation image of the vestibular apparatus is shown in (e). I experienced some difficulty in paintfilling the inner ears from *Jagged1* conditional knockout mice. Nevertheless, the lower magnification view in (d) shows the gross defects in morphology seen in the mutant mice, and the semicircular canal defect can be seen in a higher magnification image from another conditional knockout mouse in (f). Scale bars in a and b are 1mm.

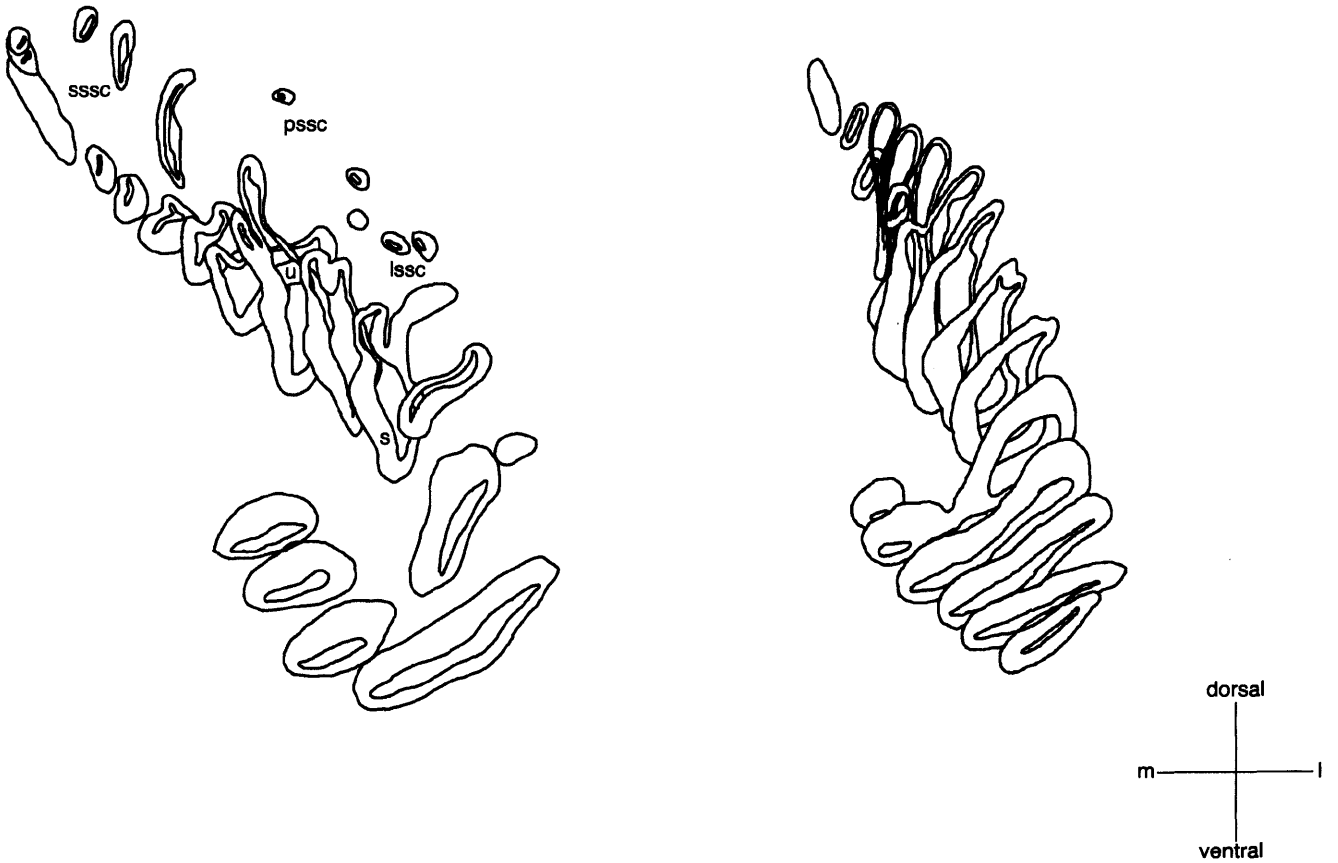


Figure 4.8

3D reconstruction of the otocyst at E13.5 in the wildtype (a) and the *Jagged1* conditional knockout cochlea. Abbreviations are: saccule (s), lateral semicircular canal (lssc), posterior semicircular canal (pssc), superior semicircular canal (sssc) and utricle (u).

Genotype	Length of cochlea (μm)	Litter Ref.
Foxg1$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	3188	Litter 1
wildtype	3881	Litter 1
Foxg1$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	2898	Litter 1
Wildtype	4088	Litter 1
Foxg1$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	1331	Litter 2
Jagged1^{flox}/+	2153	Litter 2
Foxg1$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	1254	Litter 2
Jagged1^{flox}/+	1694	Litter 2

Table 4.1

Length of the cochlea at E17.5 in *Jagged1* conditional knockout mice compared to littermate controls.

The length of the cochlea from E17.5 individuals was measured using confocal images of flat-mounted pieces of cochlea (cut into apical, middle and basal portions) and Zeiss LSM 5 Image Browser software.

Litter references are inserted to identify littermates, and do not refer to separate experiments.

Staining of these sections revealed a loss of *Jagged1* staining in all sensory patches of the *Jagged1* conditional knockout. As would be predicted on the basis of the gross morphological defects seen in these mice, several vestibular patches were absent, though the vestibular phenotype varied between individuals. In all cases, neither the anterior nor the posterior cristae were found. In one case the horizontal crista and adjacent utriclar macula could be morphologically identified, although both appeared to lack hair cells, judged from the absence of actin-stained hair bundles at the apex of cells and the loss of the bilayered structure of the sensory epithelia (Figure 4.9). The saccular macula, however, was present in this conditional knockout, and the normal structure of hair cells and supporting cells could be seen (Figure 4.10). In a second specimen, again no anterior or posterior cristae could be identified. One calretinin positive hair cell was visible in what could be morphologically identified as the horizontal crista. I could not find a utricle, though I studied a full series of sections through the inner ear. Again, the saccular macula was present, and appeared normal.

To determine whether the size of the saccular macula was altered in the *Jagged1* mutant, I measured the total volume of sensory patch epithelium (identified by the presence of hair cells) after photographing the entire sensory patch in serial sections (the size of the saccular macula was determined in one *Jagged1* conditional knockout at E17.5 and a $Foxg1^{Cre/+}$ littermate control, as described in Materials and Methods). This analysis revealed that the saccular macula was slightly reduced in size in the *Jagged1* conditional knockout (2,666,985μm^3) compared to the *Foxg1* heterozygous littermate control (2,972,220μm^3). To check whether the production of hair cells was altered in the absence of *Jagged1,* I counted hair cells in a 200μm length of sections of the patch. Sections of the saccular macula that were taken perpendicular to the epithelium were used, with three samples collected per individual, and hair cells were scored when their nuclei lay apically in the epithelium, with cytoplasm that stained positive for calretinin. The density of hair cells was slightly, but not significantly increased in the conditional knockout sacculear macula, with 37.3±3.8 (mean±SEM: n=3) hair cells, compared to 32.7±5.3 (mean±SEM: n=3) per 200μm in the control. This analysis showed that hair cells are produced in the normal density, though in a slightly smaller saccule, in the *Jagged1* conditional knockout.

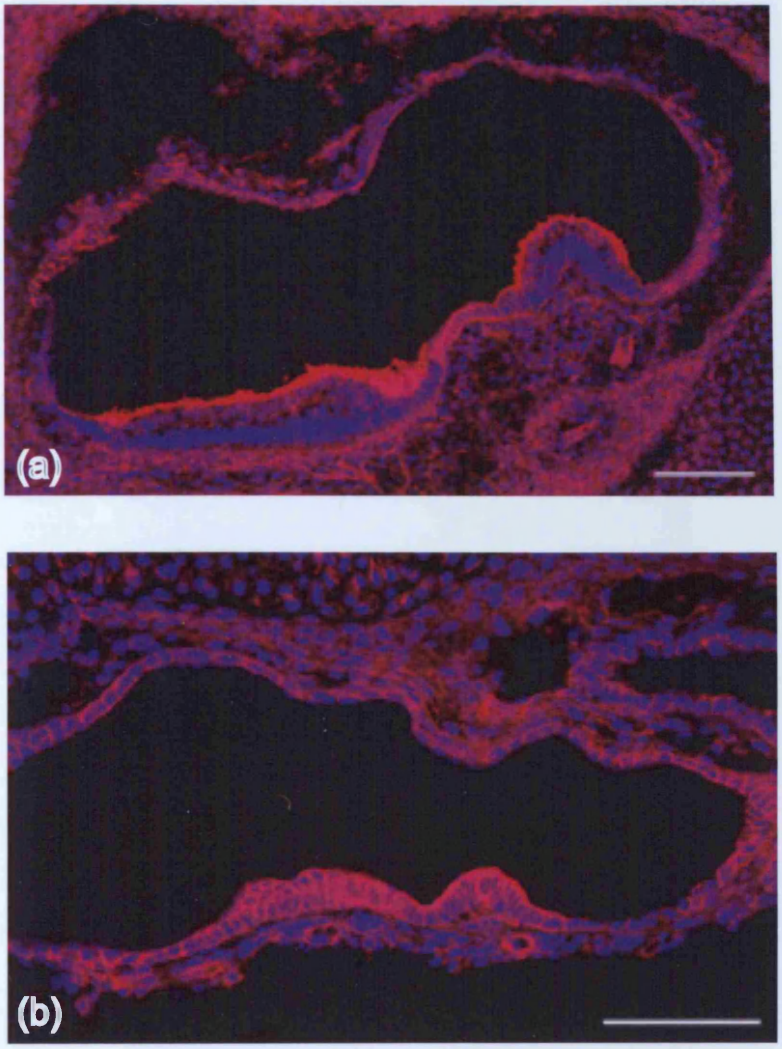


Figure 4.9

The utricular macula and the horizontal crista in a control mouse (a) and in a *Jagged1* conditional knockout mouse (b) at E17.5. Sections are stained with DAPI (blue) and phalloidin (red). Please note the difference in scale between the two images, each scale bar is 100μm.

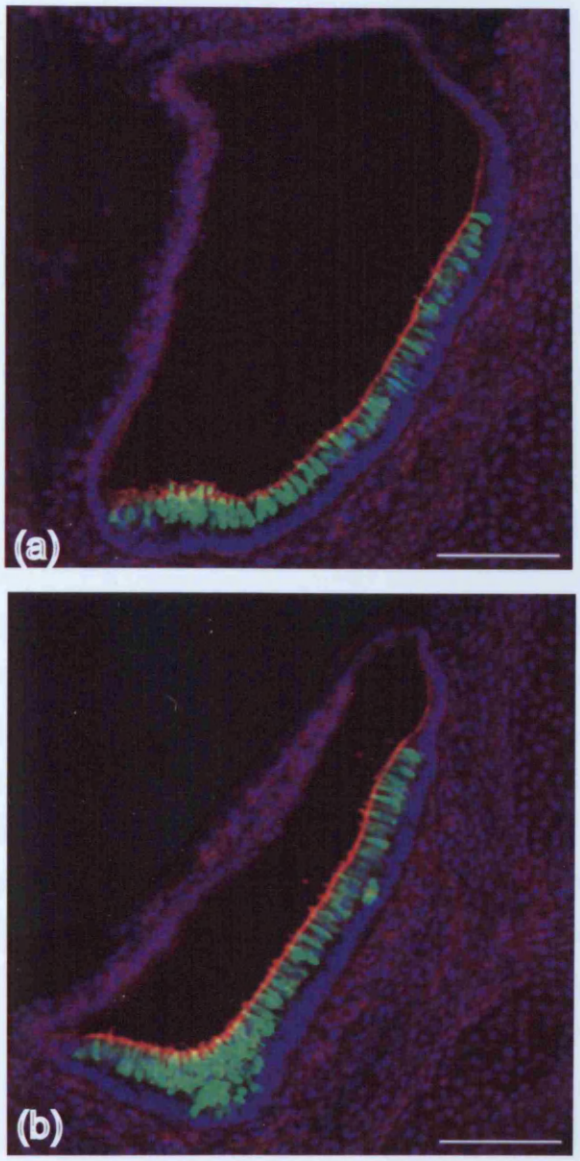


Figure 4.10

The saccular macula appears normal in the *Jagged1* conditional knockout (b) with the normal apical layer of calretinin positive hair cells (green) with actin rich apical bundles (stained red with phalloidin) and basal layer of supporting cells (seen below hair cell layer with nulcei stained with DAPI in blue).
Scale bar is 100μm.

4.2.7 Loss of *Jagged1* affects hair cell numbers, but not the timing of differentiation

I now move on to describe the effect on hair cell production seen in the *Jagged1* conditional knockout cochlea. This effect varied along the apico-basal length of the cochlea. I begin by describing that seen in the apical and middle portions.

As discussed in the introductory section of this chapter, different predictions might be made as to the effect of homozygous loss of *Jagged1*. One suggestion is that *Jagged1* expressed throughout the early sensory patch prevents premature differentiation of hair cells, and and/or helps to limit the number of hair cells produced. Its removal would then lead to premature and/or excessive hair cell differentiation. Another possibility is that it acts to specify the sensory patch, perhaps being required to propagate a signal that confers sensory patch identity. Loss of *Jagged1* would then result in a loss or reduction of the sensory patch.

To find out which, if any, of these hypotheses are correct, I started by examining the sensory epithelium of the cochlea in *Jagged1* conditional knockout mice at E17.5. The cochlea is a particularly useful organ in which to study hair cell production as many different stages of the process can be seen in one individual on the same embryonic day, because the stage of hair cell production differs along the length of the cochlea. Hair cell differentiation occurs in a wave that begins in the mid-basal part of the cochlea and travels basally and apically. This process begins at E15.5, and reaches the apex of the cochlea at early postnatal stages. Thus at E17.5 no differentiated hair cells can be seen in the apical part of the cochlea, though the immature hair cells can be identified on the basis of their raised levels of actin. The sensory patch is more mature towards the basal region so that the normal pattern of four rows of differentiated hair cells can be seen in the middle and basal portions of the cochlea at this stage, with those in the basal region displaying the more mature morphology. One can thus observe both the timing and the spatial pattern of hair cell production in the cochlea at this stage.

I collected ten conditional knockout mice and eight suitable littermate controls. Every mouse in the litter containing the mutant mice was genotyped both to confirm the genotype of the conditional knockout mouse, and to identify suitable littermate controls. The cochleas were dissected to expose the sensory epithelium and were stained with fluorescent phalloidin to mark filamentous actin. This stain labels cortical actin at the cell

boundaries, and also brightly labels the actin-rich stereocilia bundles at the apex of the hair cells. This preparation of the cochlea revealed a striking disruption of hair cell production.

In the apical part of the conditional knockout cochlea, as in the wildtype, no differentiated hair cells are found using this method of detection, that is, no hair bundles are seen. However, in both the conditional knockout cochlea and the cochlea of littermate controls, a band of cells that have upregulated their cortical actin can be seen in the apex, and can be tentatively identified as prospective hair cells. The timing of actin upregulation does not appear to be significantly altered in conditional knockouts as compared to littermate control mice (Figure 4.11). This argues strongly against the idea that *Jagged1* is required to prevent premature hair cell differentiation.

In the middle portion of the E17.5 cochlea, the normal stereotyped pattern of three rows of outer hair cells and one row of inner hair cells can be seen in the cochlea of littermate controls. In the *Jagged1* conditional knockout the number of hair cells is dramatically reduced. A continuous band of approximately two rows of hair cells is seen (Figure 4.12). These hair cells are not in regular rows, but are rather disorganised in terms of spacing from one another. They also appear to show some mis-orientation of their hair bundles, although I did not analyse this in detail. They appear to be separated from one another by intervening supporting cells, but with a wider separation between nearest-neighbour hair cells then is seen in the wildtype cochlea.

4.2.8 Half the normal number of auditory hair cells are produced in the middle part of the cochlea in the absence of *Jagged1*

In order to compare the numbers of hair cells in the conditional knockout mice to that of control individuals in a precise manner, I counted cells in phalloidin-stained wholemount cochleas. I collected that data for middle turns of the cochleas from ten conditional knockout mice, and eight corresponding control littermates. In this part of the cochlea a continuous band of hair cells is seen in the conditional knockout, as well as in the wildtype. I counted hair cells in three non-overlapping 100μm lengths of the organ of Corti for each individual, and the average number of hair cells per 100μm sample area was calculated (Table 4.2).

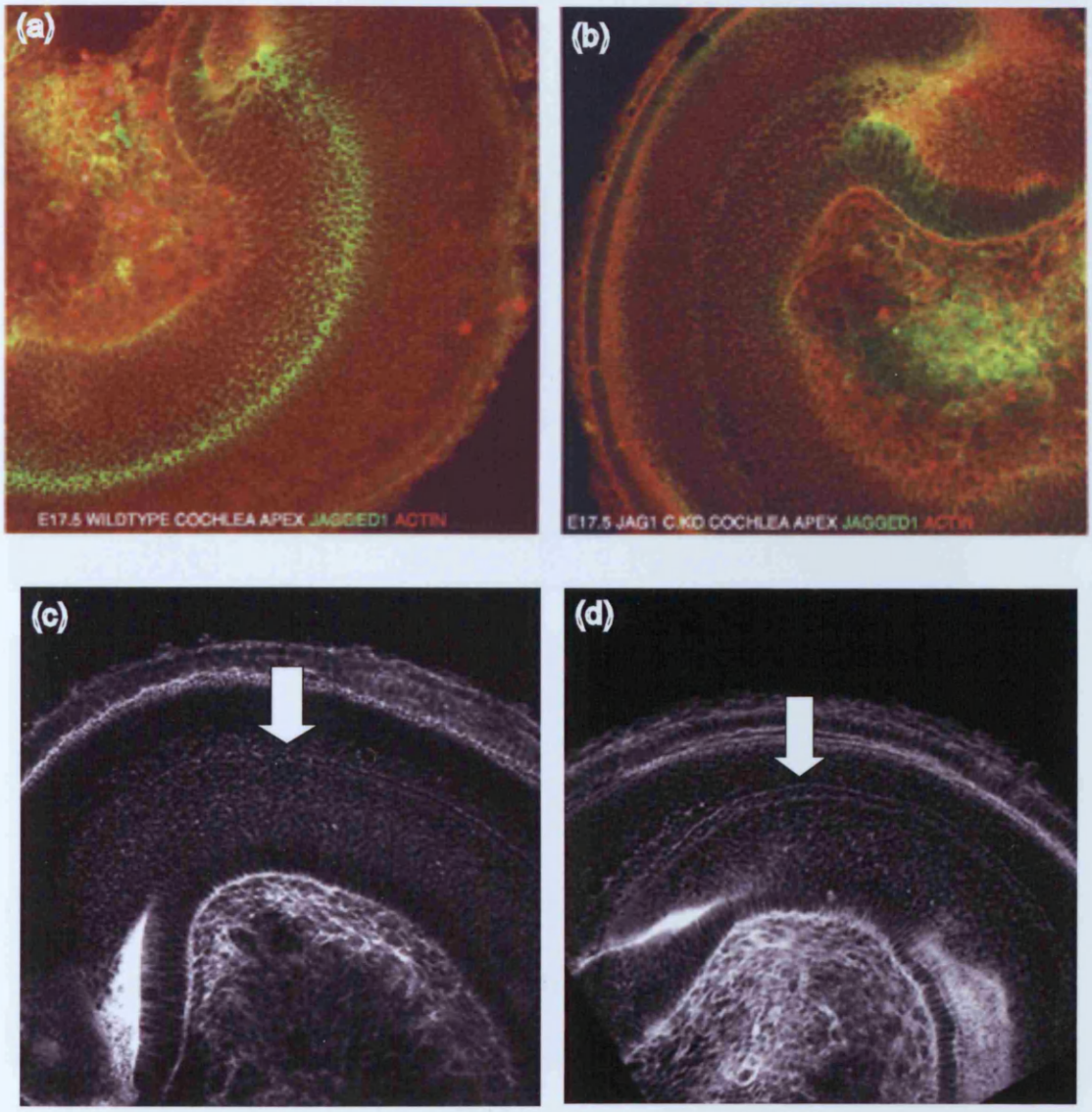


Figure 4.11

No hair cells are formed in the apex of the cochlea from both wildtype (a,c) and *Jagged1* conditional knockout mice (b,d).
(a and b) Jagged1 staining is lost in the apex of the cochlea in the conditional knockout mice. (Jagged1 antibody in green, phalloidin in red)
(c and d) A phalloidin stain reveals an upregulation of actin in the apex of the cochlea, from both wildtype (c) and mutant (d) mice outlining the developing inner hair cells (white arrows).

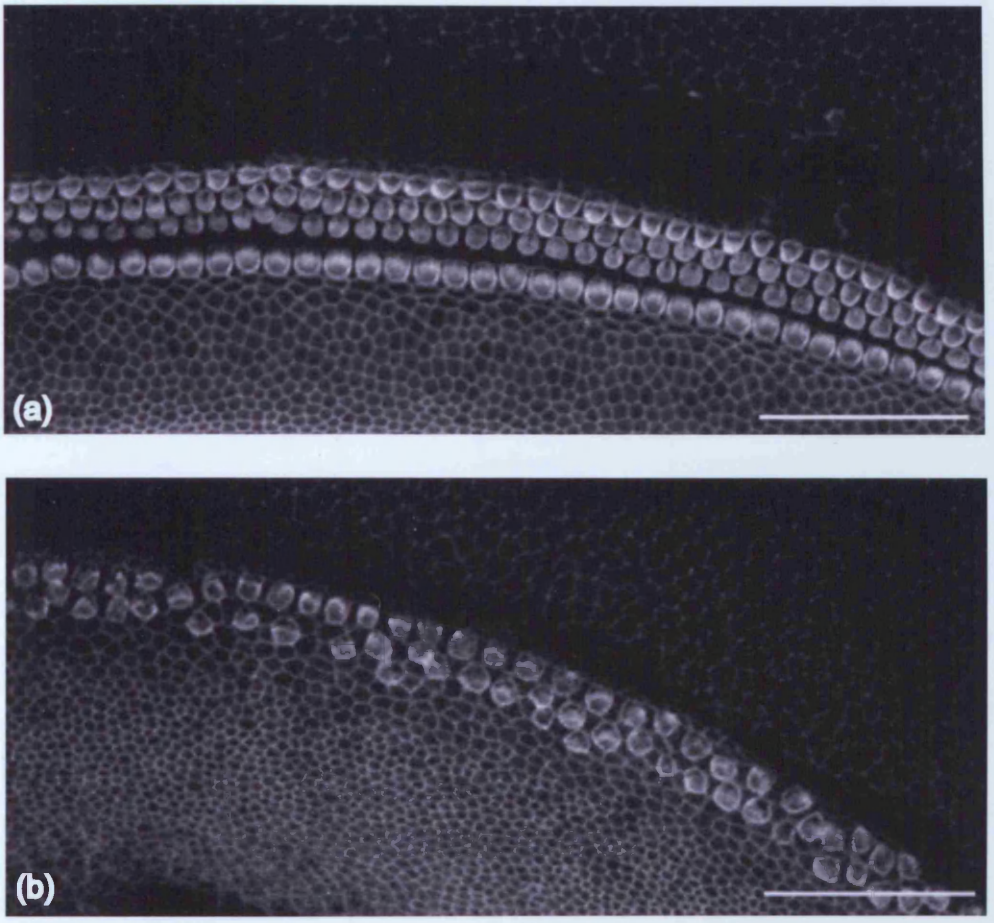


Figure 4.12

The pattern of hair cells seen in the middle part of the cochlea in the *Jagged1* conditional knockout at E17.5.
A simple phalloidin stain reveals the arrangement of three rows of outer hair cells and one row of inner hair cells in the middle aprt of the cochlea from littermate control mice (a).
In the *Jagged1* conditional knockout, approximately two disorganised rows of hair cells are formed (b).
Scale bars are 100μm.

Litter Ref.	Genotype	HC per. 100μm (of which IHCs)	HC per. 100μm (of which IHCs)	HC per. 100μm (of which IHCs)	Average over the three counts
Litter 1	*Foxg1*$^{Cre/+}$	57 (14)	58 (16)	56 (13)	57
Litter 1	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	26	23	29	26
Litter 1	*Jagged1*$^{\Delta flox/+}$	65 (15)	64 (15)	65 (15)	65
Litter 1	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	25	25	24	25
Litter 2	*Jagged1*$^{\Delta flox/\Delta flox}$	65 (15)	65 (15)	63 (15)	64
Litter 2	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	24	32	23	26
Litter 2	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	26	28	27	27
Litter 2	*Jagged1*$^{\Delta flox/+}$	64 (15)	64 (15)	65 (15)	64
Litter 3	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	25	25	25	25
Litter 3	*Foxg1*$^{Cre/+}$	71 (17)	72 (17)	71 (18)	71
Litter 4	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	21	22	22	22
Litter 4	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	24	27	25	25
Litter 4	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	29	29	25	28
Litter 4	wildtype	60 (15)	60 (15)	60 (15)	60
Litter 5	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	27	26	27	27
Litter 5	wildtype	61(16)	59 (15)	63 (16)	61
Litter 6	*Foxg1*$^{Cre/+}$:*Jagged1*$^{\Delta flox/\Delta flox}$	25	30	27	27
Litter 6	wildtype	65 (15)	69 (15)	65 (15)	66

Table 4.2

Hair cell count details performed on *Jagged1* conditional knockout mice and littermate controls.

Litter references inserted to indicate littermates, and do not refer to separate experiments.

The counts revealed that the total number of hair cells in the middle portion of the cochlea in *Jagged1* conditional knockouts is reduced by approximately 60%, with an average of 63.5 ± 1.5 (mean±SEM:n=10) hair cells seen in the normal situation, and an average of 25.8 ± 0.5 (mean±SEM:n=8) hair cells seen in the *Jagged1* conditional knockout cochlea. This finding is consistent with the initial impression that there are almost two irregular rows of hair cells, as opposed to the normal four rows. The number of hair cells per 100μm is very consistent in the normal situation of three rows of outer hair cells and one row of inner hair cells. Approximately fifteen hair cells span 100μm, with four rows giving around 60 hair cells. In the mutant there are not quite two rows of hair cells formed from around 26 hair cells.

4.2.9 *Jagged1* is required for the production of outer hair cells

I have shown that the number of hair cells in the middle part of the *Jagged1* conditional knockout cochlea is dramatically reduced, with approximately half the number of hair cells being produced in comparison to the normal situation. However, the disruption of hair cell production in the cochlea of *Jagged1* conditional knockout mice is not simply a general reduction of hair cell number. Rather, it seems as though inner hair cells are produced in excess, while outer hair cells are missing. The hair cells produced in the *Jagged1* conditional knockout appear to be in two disorganised rows of inner hair cells, or at least hair cells in the region normally populated by inner hair cells. Two lines of evidence support this impression. One is the location of these cells in the epithelium relative to other cell types. The second is the time at which they differentiate.

The supporting cells of the organ of Corti are not a homogeneous population. There are several different types of supporting cells, each with a characteristic morphology. One type is the inner pillar cells, which form a row along the length of the organ of Corti, separating the inner and outer hair cell populations. These columnar cells extend from the basement membrane to the surface of the epthelium. Their rectangular apical surfaces can be seen in a surface view of the organ of Corti stained with fluorescent phalloidin. The cell body narrows and extends to the basement membrane, where it forms a wide footplate. In the *Jagged1* conditional knockout mice, a row of cells with morphology typical of inner pillar cells can be seen lying outside the disorganised rows of hair cells in the *Jagged1* conditional knockout cochlea. This row of cells was seen in all cases (n=5) where z-stacks

of the developing sensory patch were taken using the confocal microscope, and they always lay on the outer side of the two rows of hairs cells that were present (Figure 4.13).

The second line of evidence that the remaining hair cells in the *Jagged1* conditional knockout cochlea are inner hair cells comes from calretinin staining. In addition to the basal-to-apical wave of differentiation of hair cells in the cochlea, differentiation also spreads radially, so that at any given position along the length of the cochlea inner hair cells begin to differentiate first, and the three rows of outer hair cells are formed sequentially. This sequence of hair cell production is reflected in the pattern of staining observed with an anti-calretinin antibody. Calretinin is first seen in the inner hair cells, then in the rows of outer hair cells, acccording to their timing of differentiation. Thus, in the middle part of the wildtype mouse cochlea at E17.5, calretinin antibody staining marks inner hair cells, but is not yet visible in the outer hair cells. Calretinin is also seen in the row of pillar cells. This pillar cell staining in the normal cochlea was unexpected, as calretinin is widely used as a marker of hair cells. However, it is consistent with the finding that non-sensory cells at the border of vestibular patches in the rat inner ear stained positive with the same calretinin antibody (Zheng and Gao 1997).

The pattern of calretinin staining in the middle part of the cochlea from E17.5 *Jagged1* conditional knockout mice is shown in Figure 4.14. All the hair cells in the *Jagged1* conditional knockout stain positive for calretinin throughout the cytoplasm, with a single row of pillar cells stained just outside them. In control mice, the single row of inner hair cells stains positive,again with one row of calretinin-positive pillar cells just outside them, and no staining is yet seen in the outer hair cell rows. Thus, the cochlea hair cells seen in the *Jagged1* conditional knockout resemble inner hair cells in the timing of their development as well as in their spatial relation to inner pillar cells.

In some cases in the conditional knockout there are occasional small clumps of extra calretinin positive cells outside the row of inner pillar cells. These cells are in the area where outer hair cells are normally formed, but they do not show hair bundles. They do contain plentiful actin, as do immature hair cells, but they do not resemble hair cells in the morphology of their cell bodies, and they lack apical bundles. They could perhaps be abnormal supporting cells.

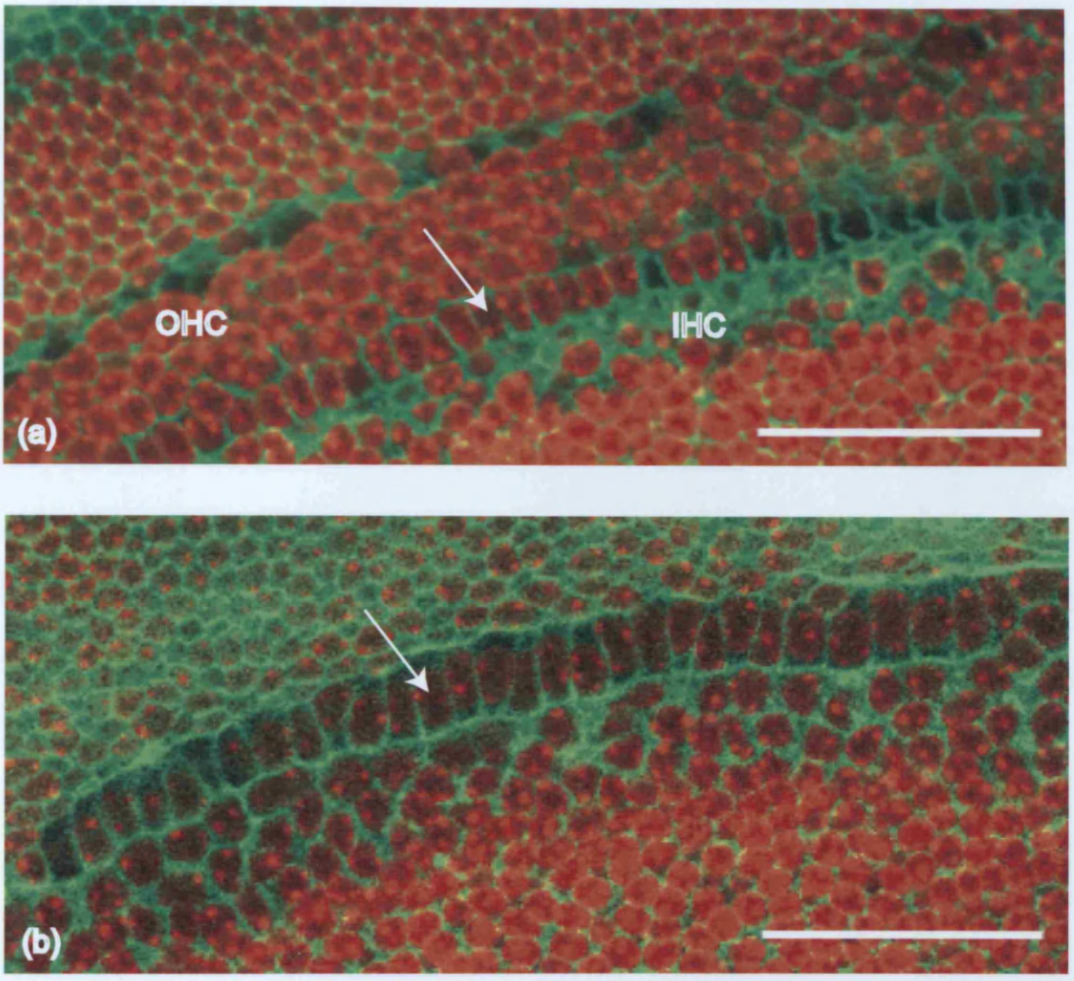


Figure 4.13
Morphological identification of inner pillar cells in the *Jagged1* conditional knockout.
Wholemount preparations of cochleas from E17.5 wildtype (a) and *Jagged1* conditional knockout (b) mice were stained with phalloidin (green) and DAPI (red).
Confocal images taken deep in the epithelium reveal cells with the morphology of inner pillar cells (arrows) lying between the outer hair cells (OHC) and inner hair cells (IHC) in the wildtype, and lying outside the hair cells in the conditional knockout cochlea. Scale bars are 50μm.

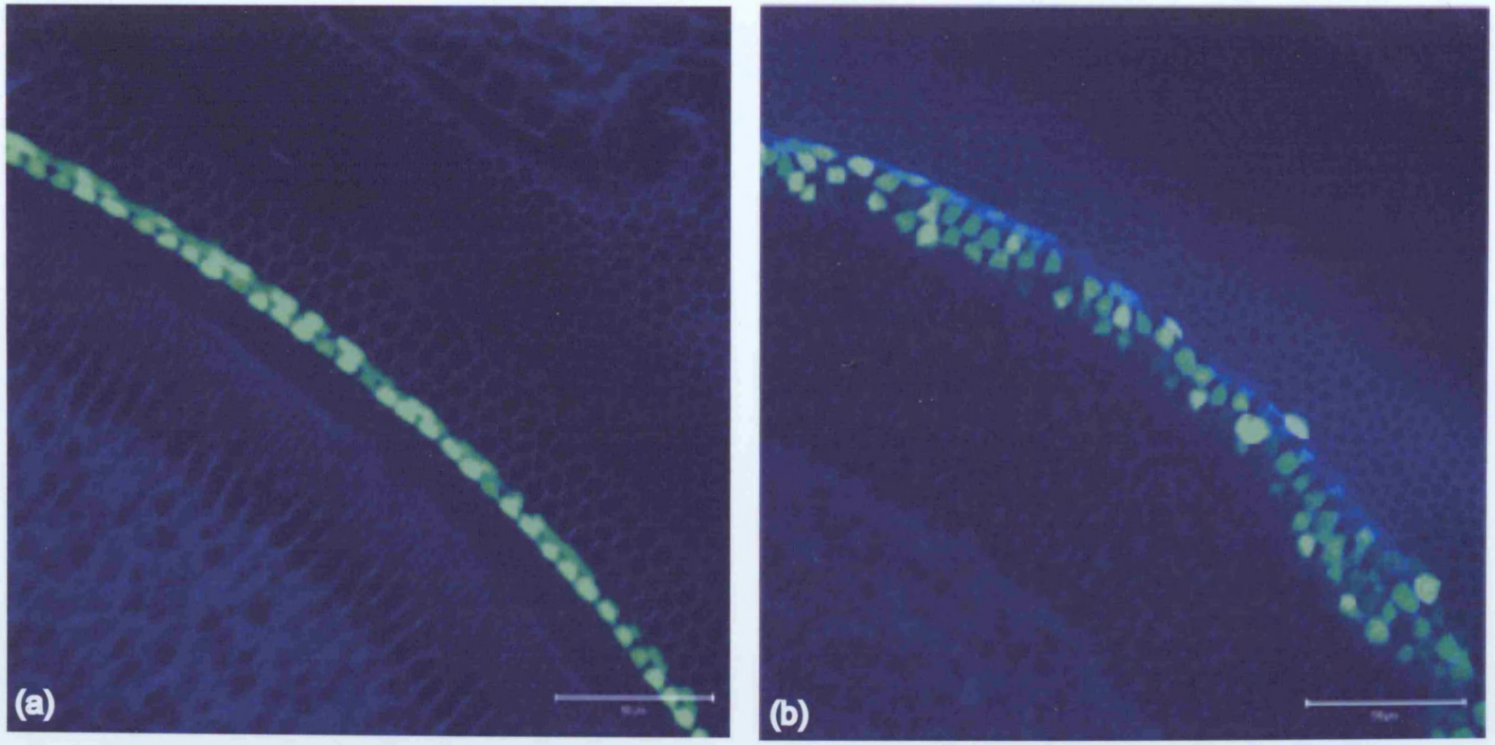


Figure 4.14
Calretinin staining (green) in the wildtype cochlea at E17.5 in the middle turn (a) stains inner hair cells and inner pillar cells throughout their cytoplasm.
In the middle part of the *Jagged1* conditional knockout cochlea at this stage (b), all hair cells produced are stained with the antibody. In addition, cells lying lateral to the hair cells stain positive with this antibody
Scale bars are 50μm.

4.2.10 Hair cell production is more severely affected in the basal part of the cochlea of *Jagged1* conditional knockout mice

The severity of the defect in hair cell production varies along the apico-basal axis of the cochlea in the *Jagged1* conditional knockout, with the loss of hair cells becoming more severe in the basal part of the cochlea.

In wildtype mice, the normal pattern of four rows of hair cells extends to the basal terminus of the cochlea at E17.5. In the *Jagged1* conditional knockout cochlea, the band of hair cells becomes discontinuous in the mid-basal region and islands of hair cells are formed (Figure 4.15). Further towards the basal end of the cochlea duct, the epithelium completely lacks hair cells.

Cell counts in the basal region of the cochlea in which hair cells were present were performed as for the middle region of the cochlea. The number of hair cells per. 100μm varied greatly in the basal region of the cochea, with some areas having approximately the same number of hair cells as seen in the middle region, and others with reduced numbers (Table 4.3). The pattern of hair cells in the basal cochlea is thus not simply a rearrangement of the two rows of hair cells seen in the middle part of the cochlea.

The first E17.5 litters collected were used for SEM analysis, but with little success due to difficulty in removing the tectorial membrane, which at this stage is closely adherent to the developing sensory patch. I therefore changed my strategy for analysis to observing fluorescently labelled wholemount cochlea preparations using the confocal microscope. This proved to be a far more efficient approach, allowing observation of the entire depth of the epithelium, regardless of whether the tectorial membrane had been removed successfully, as illustrated with pictures shown earlier. Nevertheless, a small number of the SEM specimens were useful and images from a pair of these specimens are shown in Figure 4.16 and Figure 4.17. They show the pattern and morphology of the hair cells in the mid-basal part of the cochlea in a *Foxg1-Cre* heterozygous control individual, and in the corresponding region of a *Jagged1* conditional knockout littermate, where hair cells are arranged in an island, amid epithelium that lacks hair cells.

As the mice die shortly after this stage, I am unable to confidently distinguish between inner and outer hair cells on the basis of morphology, which becomes markedly different

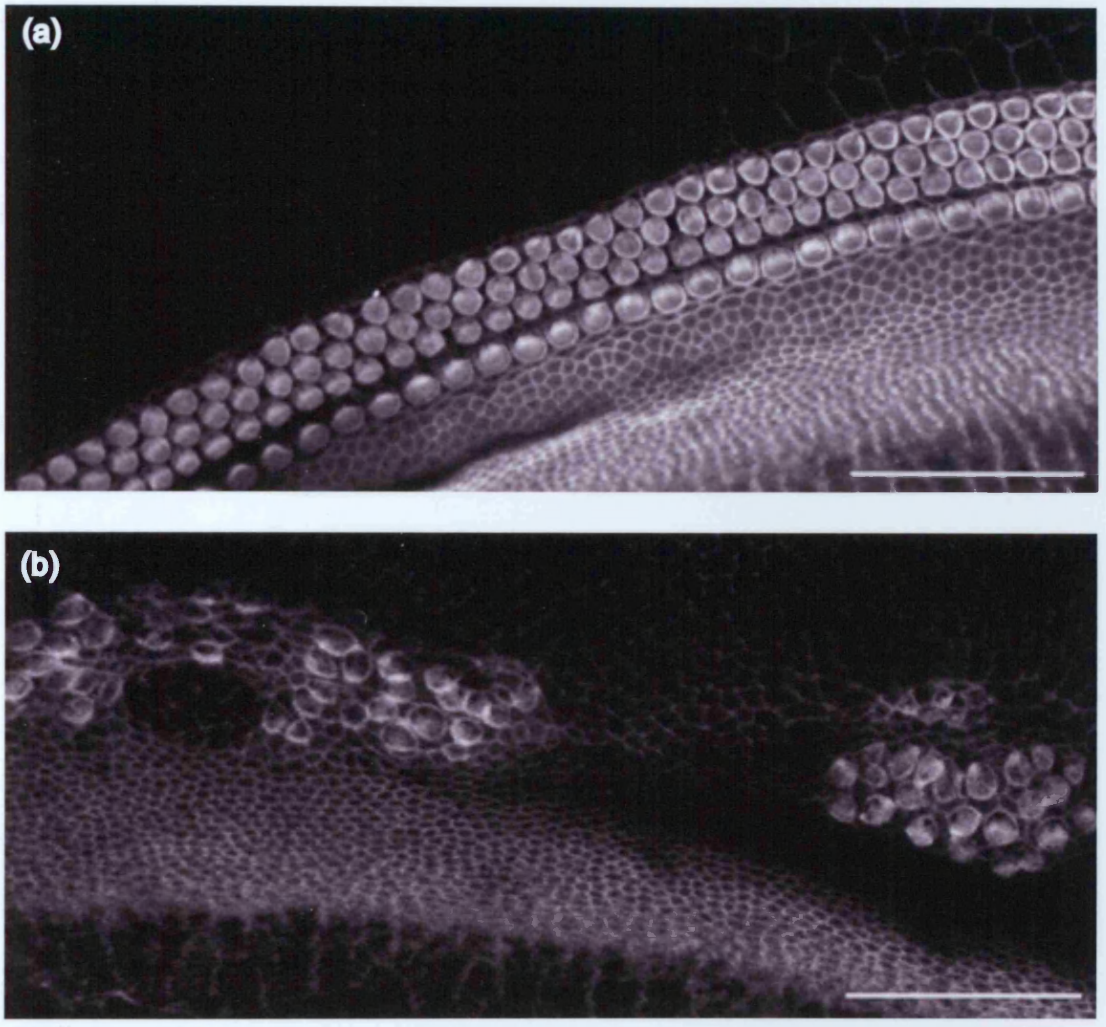


Figure 4.15

The pattern of hair cells in the basal part of the cochlea from *Jagged1* conditional knockout mice at E17.5.

Confocal images of wholemount cochleas stained with phalloidin.
In the basal part of the cochlea from littermate control mice the normal pattern of four rows of hair cells can be seen (a). In the *Jagged1* conditional knockout, islands of hair cells are formed (b), and the basal most region completely lacks hair cells. Scale bars are 50μm.

Litter Ref.	Count 1 HC per. 100μm	Count2 HC per. 100μm	Count3 HC per. 100μm	Average over the three counts
Litter 1	19	26	42	29
Litter 2	26	28	27	27
Litter 3	16	17	12	15
Litter 3	10	13	13	12

Table 4.3

Counts of hair cells per 100μm length of the cochlea in the basal region at E17.5.

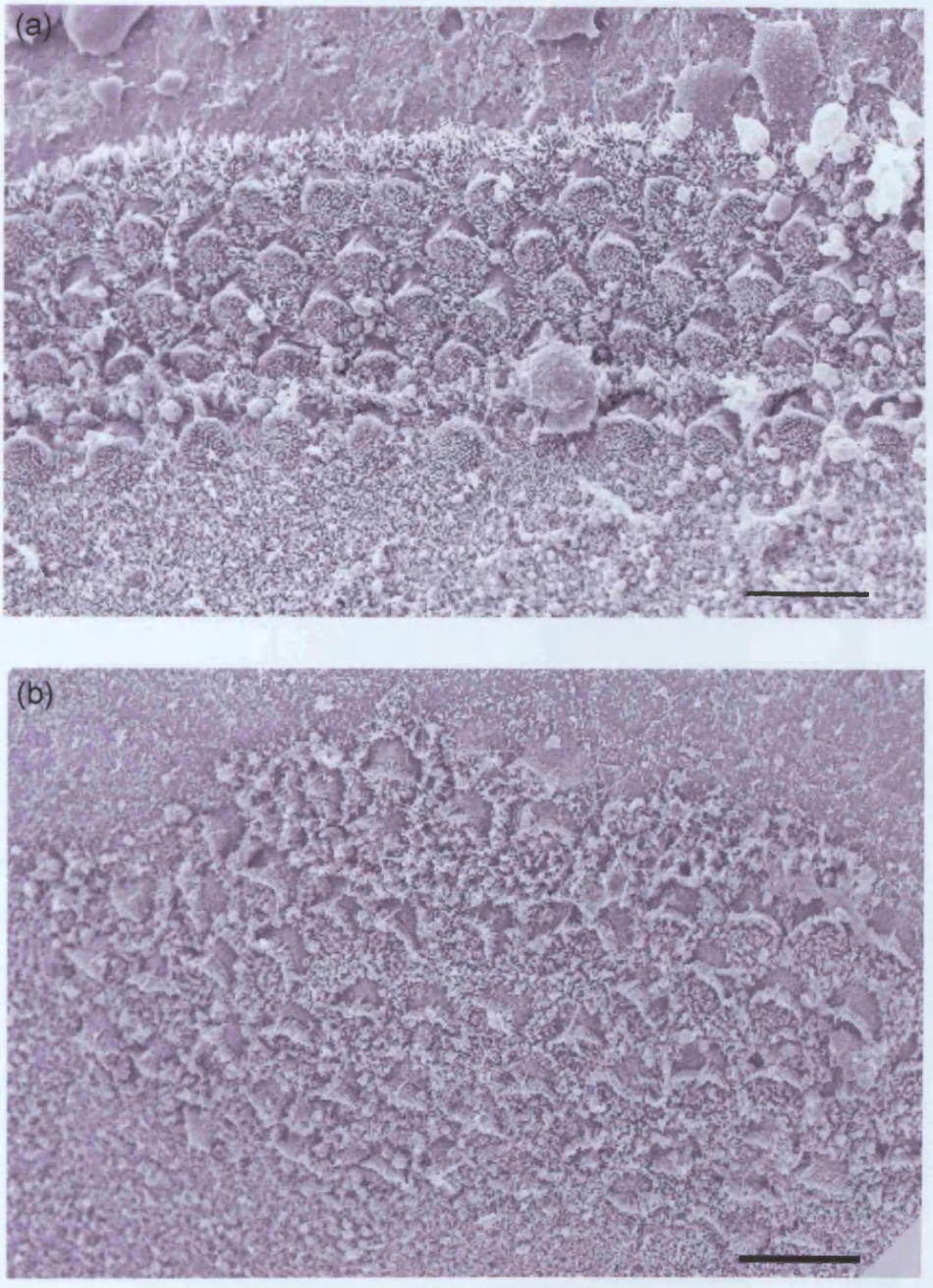


Figure 4.16
SEM showing hair cell pattern in the littermate control *Foxg1-cre* heteroygous cochlea in the midbasal region at E17.5 (a), and in the *Jagged1* conditional knockout cochlea in the same region. Scale bars are 10μm. In both cases, the medial edge of the cochlea is at the bottom of the image.

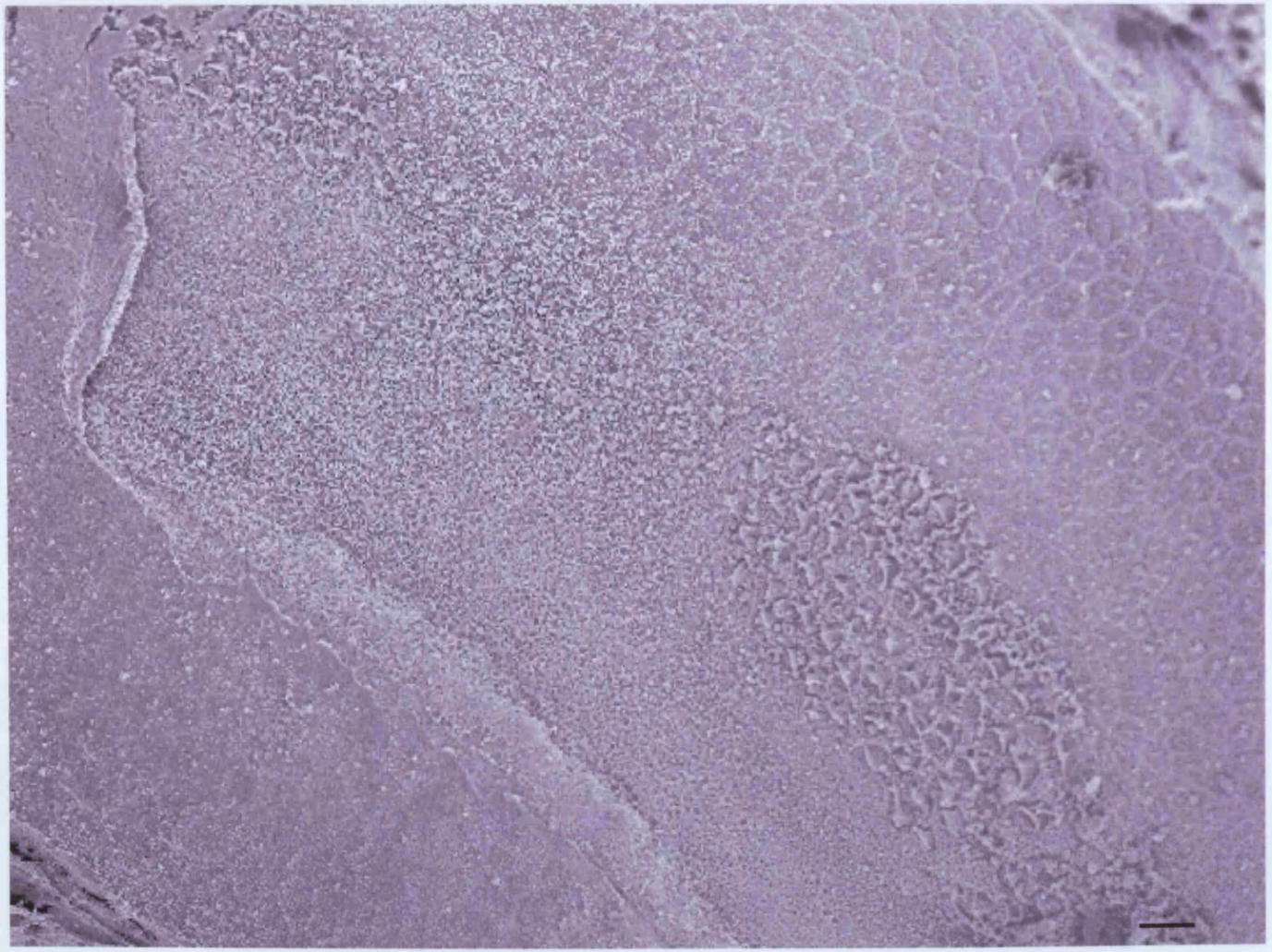


Figure 4.17
SEM of E17.5 *Jagged1* conditional knockout, showing the islands of hair cells in the mid-basal region of the cochlea. Scale bar is 10μm.

only later. Other evidence for the identity of the cells produced in the *Jagged1* conditional knockout are presented earlier in this chapter.

4.2.11 The same contrast between basal and more apical regions is seen at early and late stages

One interpretation of the difference in the severity of the hair cell patterning defects along the basal-to-apical axis of the cochlea could be that the band of cells partially degenerates as development proceeds. The mid-basal region of the cochlea is the first to differentiate, and it is conceivable that irregular degradation of the patch follows an initially orderly wave of differentiation. To test this idea I examined cochleas from conditional knockout mice at earlier stages.

At E15.5, developing hair cells can already be visualised in wholemount preparations of the normal cochlea in which actin has been stained with fluorescent phalloidin. Immature hair cells upregulate their levels of actin and thus can be identified in the basal cochlea as early as E14.5 (McKenzie, Krupin et al. 2004). A day and a half later, rows of outer hair cells can be identified in the normal cochlea in this region in a similar way.

In control mice, four rows of hair cells were seen throughout the basal portion of the cochlea, in agreement with the published account. In the *Jagged1* conditional knockout mouse, the basal most part of the cochlea at E15.5 was devoid of hair cells, and this area of epithelium devoid of hair cells made up the basal third of the entire cochlea. In the middle third of the cochlea the areas of naked epithelium were interrupted by islands of hair cells (Figure 4.18). More apically these islands fused to form a continuous band of immature hair cells, as seen at later stages.

4.2.12 *Jagged1* is required for normal levels of $p27^{Kip1}$ protein

It is possible that the decrease in the number of hair cells seen in the *Jagged1* conditional knockout cochlea is due to a reduction in the size of the prospective sensory patch early in ear development. To investigate this idea I stained specimens with an antibody against $p27^{Kip1}$, which has been described as a marker of the early sensory patch, $p27^{Kip1}$ (Chen,

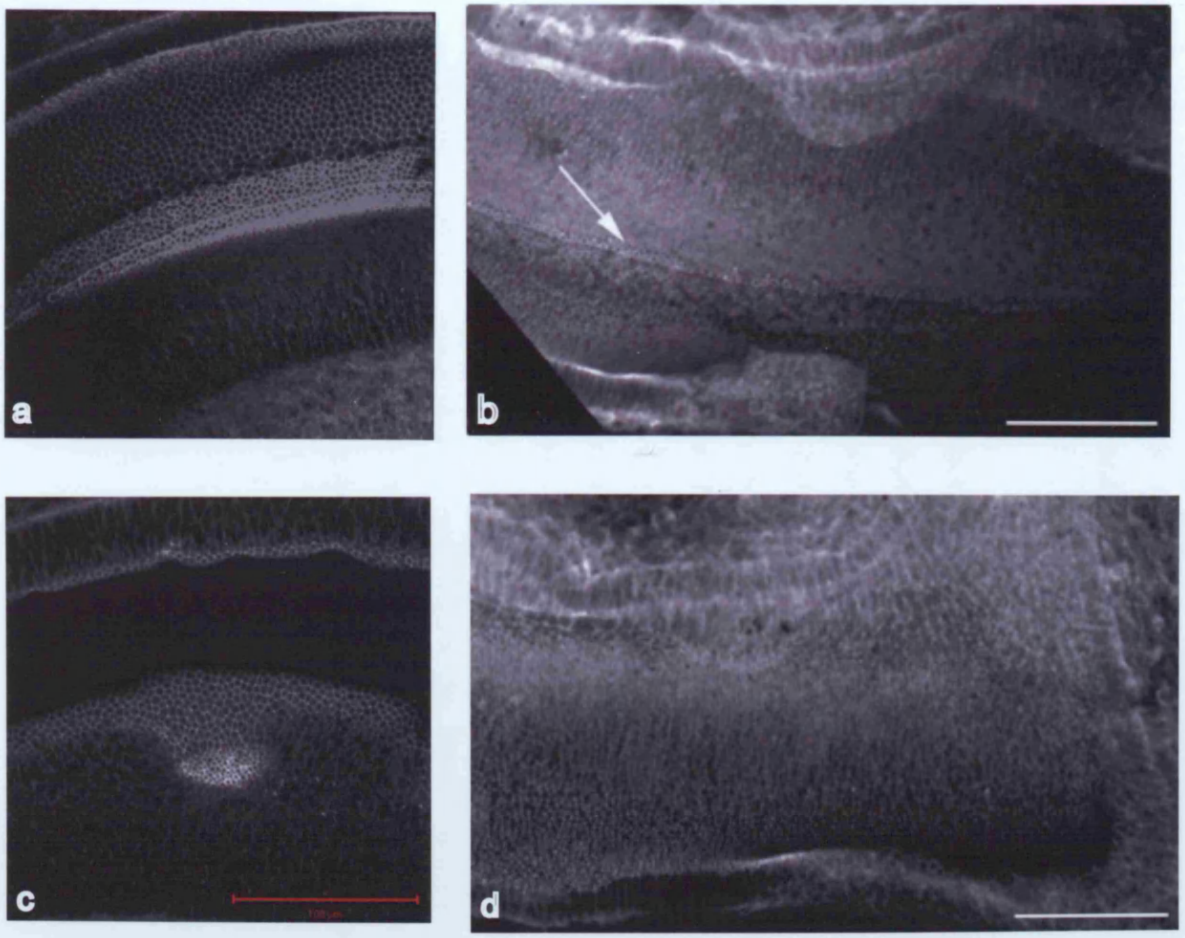


Figure 4.18
Confocal images of the wholemount cochlea at E15.5 stained with phalloidin. In (a) the normal pattern of hair cells can be seen to start differentiating the in the basal cochlea of wildtype mice. This can be seen in the basal terminus, with the single row of inner hair cells indicated (arrow) (b). In the *Jagged1* conditional knockout cochlea, an island of hair cells is seen in the basal region (c), whereas no hair cells can be seen in the basal terminus of the cochlea. Scale bars are 100μm. Scale bar in c also applies to a.

Johnson et al. 2002). $p27^{Kip1}$ marks the group of cells which exit the cell cycle in synchrony, before contributing towards the sensory patch. It is first detected in the immature sensory patch at E13.5. As cells of the sensory patch begin to differentiate it is excluded from the hair cells and restricted to the supporting cell population (Chen, Johnson et al. 2002). $p27^{Kip1}$ has an additional interest on account of its function: it is an inhibitor of the cell cycle, and thus a regulator of cell proliferation, and a possible mediator of the effects of Jagged1 on this aspect of cell behaviour. For example, one might wonder whether the deficit in hair cells in the *Jagged1* mutant could reflect a failure of cell proliferation due to overexpression of $p27^{Kip1}$.

I have used a polyclonal antibody raised against a synthetic peptide that mimics part of the human $p27^{Kip1}$ protein (Cell Signalling). This antibody has been used previously for immunostaining of wholemount cochlea (McKenzie, Krupin et al. 2004), and gives a pattern of staining similar to that published using another anti-$p27^{Kip1}$ polyclonal antibody (Chen, Johnson et al. 2002). I studied cochleas from *Jagged1* conditional knockouts and littermate controls at E14.5, when the anti-$p27^{Kip1}$ antibody stains a band of cells extending from the base to the apex of the cochlea. This band is broader at the extreme apex of the cochlea, and has become excluded from the more mature developing hair cells at the base of the cochlea. $p27^{Kip1}$ was lost or was dramatically reduced in the cochleas from *Jagged1* conditional knockout mice (n=4), though high levels of fluorescence were seen in the same pattern in all the control littermates (Figure 4.19). The remarkable and unexpected conclusion, therefore, is that Jagged1 is normally required to promote expression of the cell cycle inhibitor $p27^{Kip1}$, but that in the absence of $p27^{Kip1}$ the number of hair cells is not increased but reduced.

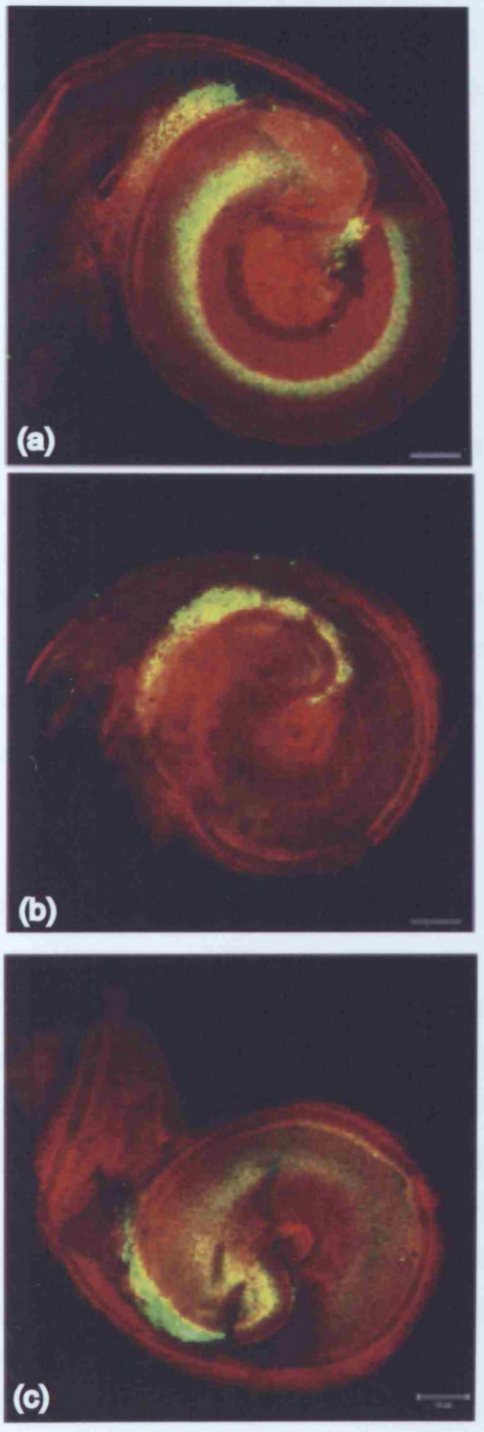


Figure 4.19

p27Kip1 antibody staining (green) in the wildtype cochlea at E14.5 marks a broad stripe of cells along the length of the cochlea. In the *Jagged1* conditional knockout, this staining is lost (b) or reduced (c) at this stage.
Wholemount cochleas are counterstained with phalloidin (red).
Scale bars are 100µm.

4.3 Discussion

In this chapter, I have shown that loss of *Jagged1* results in loss of some sensory patches and reduction in the total numbers of hair cells. The severity of the effects varies from region to region, and in one region - the region of inner hair cells - a seemingly opposite effect is seen: here, the number of hair cells is increased. In the cochlea, the alteration of hair cell numbers is accompanied by a loss of expression of the cell cycle inhibitor $p27^{Kip1}$. I will first discuss the general conclusions that can be drawn, and then examine some specific aspects of the data in more detail.

4.3.1 The vestibular patches differ from one another in their requirement for *Jagged1*

All the sensory patches normally express *Jagged1* in a similar way. It was therefore a surprise to find that loss of *Jagged1* affected them very differently. I will first summarise my data for the development of vestibular patches in the *Jagged1* conditional knockout, then discuss the general conclusions that can be drawn.

Some vestibular patches were lost in the absence of Jagged1

Loss of *Jagged1* has the most severe effect upon the sensory patches of the cristae. All *Jagged1* conditional knockout mutants analysed had a complete loss of the anterior semicircular canal, and a severe truncation of the posterior and horizontal semicircular canals. There is some evidence that loss of the canals occurred as a consequence of loss of the associated crista. Fgfs in the developing sensory patches are thought to positively regulate Bmp activity in a canal genesis zone, which lies adjacent to the patch (Chang, Brigande et al. 2004). Loss of this signal upon loss of the sensory patch would thus result in a failure of canal formation.

It is puzzling that loss of *Jagged1* had the most severe effect upon the cristae as these patches do not express *Jagged1* early in their development, though they are already distinguished by virtue of *Bmp4* expression (Morsli, Choo et al. 1998). The patch of *Jagged1* expression in the ventral otocyst appears to correlate to the patch of *Lfng*

expression, marking the early maculae and cochlea. By contrast, for the maculae and organ of Corti, the onset of *Jagged1* expression is seen at roughly the same time as *Lfng* expression, and *Bmp4* expression switches on only later (Morsli, Choo et al. 1998). Thus, the variation in the severity of the phenotype probably reflects different developmental programmes underlying the development of the different patches, indicated by the differing expression patterns of genes involved in the process, and the specific loss of different patches seen upon disruption of gene function.

Some patches seemed to be present, but failed to produce hair cells

The utricular macular and the horizontal crista were also severely affected by the loss of *Jagged1*. The utricular macular was either missing entirely, or was morphologically identifiable but lacked hair cells. Similarly, the horizontal crista was identifiable, with the usual saddle-shaped morphology typical of cristae, but appeared to lack hair cells. This phenotype superficially resembles the *Math1* null phenotype, where sensory patches that lack hair cells are also seen: all sensory patches in the ear can be identified, but they do not contain hair cells. In this case, hair cells appear to be selected from the pool of progenitors, but they fail to differentiate (Bermingham, Hassan et al. 1999; Chen, Johnson et al. 2002).

I cannot say, from my experiments, whether hair cells are completely absent, or whether the epithelium I see is prosensory in character. This would require the use of hair cell markers such as calretinin, and non-hair cell markers for the maculae and cristae, such as Lfng and Bmp4 (Morsli, Choo et al. 1998).

Based upon the appearance of the tissue, however, it appears that the patches may have formed but failed to produce hair cells. One explanation is that *Jagged1* is required at a late stage for differentiation of hair cells and/or maintenance of the prosensory patches in some areas of the ear.

One patch developed normally in the absence of Jagged1

The saccule was present in both of the *Jagged1* conditional knockout mice in which the development of the vestibular patches was analysed in detail. In both cases it was slightly

reduced in size, although it is possible that this reduction in the size of the sensory patch is a reflection of the reduced size of the whole inner ear and of the embryo itself, compared to littermates. The arrangement of hair cells and supporting cells in the epithelium did not appear to be disrupted, the two layers of cells were clearly visible, and the density of hair cells unchanged. This is interesting in the light of results looking at hair cell density in the vestibular patches in *Hes* mutant mice, where there was an increase in the number of hair cells per. unit length of the epithelium in both *Hes1* and *Hes5* null mice. A further increase in the number of hair cells was observed in compound mutants (Zine, Aubert et al. 2001). I found no such increase in the number of hair cells in the saccule from *Jagged1* mutant mice, suggesting that *Jagged1* is not required for the expression of *Hes1* and *Hes5* in inhibiting production of hair cells within the developing sensory patches.

The role of Jagged1 development of the vestibular patches

While the effect of loss of *Jagged1* varied widely between different vestibular patches, general conclusions may be drawn as to its normal function. It appears that Jagged1 has two roles, but is not required everywhere for them: (1) an "early " role in specifying sensory patches, and (2) a "late" role in controlling differentiation within these patches.

It is striking that I did not see an overproduction of hair cells in the vestibular patches. It is possible that hair cells may have been overproduced and then died and been removed, but I saw no sign of such a phenomenon at any stage. A key conclusion from my data, therefore, is that Jagged1 does not simply mediate inhibition of hair cell differentiation. This appears to rule out one of the main initial theories for the role of Jagged1 in inner ear development.

Thus, my data suggest that Jagged1 helps in specification of sensory patches and subsequently in maintaining the competence of precursors within them to differentiate as hair cells. It is not excluded, however, that Jagged1 might at the same time exert some inhibition over the final step of hair cell differentiation, as I shall discuss below.

4.3.2 *Jagged1* in the cochlea, as in the vestibular regions, helps specify and/or maintain the prosensory patch

The sensory epithelium of the cochlea in *Jagged1* conditional knockout mice is dramatically reduced, with a severe reduction in the number of hair cells, even considering the reduction in the overall size of the inner ear in the mutant mice. The organ of Corti in the middle part of the cochlea contains about half the number of hair cells seen in the normal cochlea in this region. In the most basal part of the cochlea no hair cells are formed. Islands of hair cells are seen in the mid-basal region before a continuous band of about two rows of hair cells are seen in the middle part of the cochlea. This reduction in the size of the patch of hair cells implies that *Jagged1* in the cochlea, as in the vestibular patches, is needed for correct specification and/or maintenance of the sensory patch.

There is, however, a striking difference between the effects on the inner and outer hair cell populations. Loss of *Jagged1* appears to have opposite effects upon the different hair cell types, causing an increase in the number of inner hair cells, whereas outer hair cells seem to be entirely lost. There are at least two ways of interpreting this. (A) One would propose that loss of *Jagged1* has a severe effect on the size of the sensory patch, to the extent that only inner hair cells are produced, and that these cells are subsequently produced in excess. (B) The other would propose that the net effect of loss of *Jagged1* is to cause a slightly less severe reduction in the number of hair cells, and would suggest that loss of *Jagged1* in addition has shifted the pattern of specification of inner versus outer hair cell character. In other words, some cells that would display an outer hair cell character are converted to inner hair cells in its absence.

Hypothesis (A) seems the simplest, and, so far as the inner hair cell effects, would fit better with the observations of Zine et al (2000), who saw an increase of hair cell numbers in cochlear explants treated with *Jagged1* antisense oligonucleotides (Zine, Van de Water et al. 2000). The phenotype in the outer hair cell region would reflect the primary importance of the early function in that region, while the excess of inner hair cell production would reflect the late function. On the other hand, hypothesis (B) proposes a novel function for Jagged1.

It is not clear how Jagged1 might perform its "later" role, controlling hair cell production. The obvious suggestion is that Jagged1 normally inhibits hair cell differentiation, performing a role that is well established for Notch signalling in the inner ear. Another theory is that the later role of Jagged1 is to inhibit proliferation in the sensory patch. While this theory proposes a novel role for Notch signalling in the inner ear, it receives support from my findings with regard to $p27^{Kip1}$. This cell-cycle inhibitor, which has been shown to limit proliferation of cochlea sensory patch cells in other experiments (Chen, Johnson et al. 2002), is strikingly downregulated in the *Jagged1* conditional knockout cochlea. Though more extensive analysis at other stages is required to draw definite conclusions, the preliminary findings are provocative. From present data, it is hard to distinguish between the possibility that inner hair cells are overproduced because of excess proliferation, or because of a loss of Notch-mediated inhibition.

4.3.3 There are some aspects of the *Jagged1* conditional knockout that require further analysis

Is the prosensory patch initially reduced in the Jagged1 conditional knockout cochlea?

While there are certainly fewer hair cells produced in the *Jagged1* conditional knockout cochlea, it is not clear from my experiments whether this reflects a reduction in the size of the prosensory patch, or whether the prosensory patch has failed to mature correctly to produce hair cells. It would be interesting to look at the expression patterns of *Bmp4* and *Lfng* in the *Jagged1* conditional knockout cochlea. In the cochlea at P1, *Bmp4* is expressed just outside the sensory patch, in the population of Hensen's and Claudius' cells. If the sensory patch is indeed severely reduced in the *Jagged1* conditional knockout, it would then be important to differentiate between the possible mechanisms by which this occurred. Did the prosensory patch form normally, but then degenerate, or does the patch fail to enlarge in the absence of *Jagged1*? My data argue against a progressive degeneration of the patch, but do not rule out regionalised degradation of the patch, or a failure of the patch to enlarge.

Are the hair cells produced in the absence of Jagged1 all inner hair cells?

Another aspect of the *Jagged1* conditional knockout phenotype that requires further analysis is the identity of the hair cells observed in the cochlea. These cells all have characteristics of inner hair cells. Specifically, they all switch on expression of a hair cell marker, calretinin, at a time when only inner hair cells do so in the normal cochlea (Dechesne, Rabejac et al. 1994). Also, their location in the epithelium in relation to other cell types is typical of inner hair cells: they lie medial to a row of inner pillar cells, which in the normal cochlea separate the row of inner hair cells from the first row of outer hair cells. I have not shown conclusively, however, that these cells are inner hair cells by the criterion of hair-bundle morphology or function.

The published account of the *Jagged1* heterozygous phenotype in the cochlea (described in *Headturner (Htu)* and *Slalom (Slm)* mice) casts doubt upon the assertion that only inner hair cells are produced in the *Jagged1* conditional knockout (Kiernan, Ahituv et al. 2001; Tsai, Hardisty et al. 2001). In both *Htu* and *Slm* heterozygotes there is a decrease in the number of outer hair cells seen in the cochlea, and an increase in the number of inner hair cells, or at least of hair cells in the inner region. As heterozygous *Jagged1* mutant mice survive to adulthood, the mature morphology of the hair cells could be observed using SEM. This showed that some of the extra hair cells in the inner region had a W-shaped morphology, typical of outer hair cells. It remains possible that some or all of the hair cells produced in my *Jagged1* conditional knockout mutant are outer hair cells in the inner region.

4.3.4 *Jagged1* is not required to prevent premature hair cell production

Though the production of hair cells is severely disrupted in terms of numbers and arrangement, the timing of hair cell production does not appear to be disrupted in the *Jagged1* conditional knockout. Thus rules out the hypothesis that the function of early Jagged1 in the prosensory patch is to prevent premature differentiation.

The absence of premature differentiation argues against a role for *Jagged1* in inhibiting hair cell differentiation at early stages in the development of the prosensory patch, but it

remains possible that Jagged1 acts later to control hair cell production, as discussed earlier.

4.3.5 *Jagged1* is not required for production of supporting cells.

The hair cells produced in the *Jagged1* conditional knockout cochlea are not all in contact with one another, but are separated by intervening cells. Also, cells with the characteristic morphology of inner pillar cells are seen in the *Jagged1* conditional knockout. This suggests that *Jagged1* is not required for establishing the alternating pattern of hair cells and supporting cells. It is not required in the supporting cell population to prevent them from forming hair cells, nor for adopting the supporting cell fate.

This finding appears to be at odds with the idea that Jagged1 acts late in sensory patch development to inhibit hair cell production. However, as I shall discuss in chapter 5, there are other possible explanations for this.

4.3.6 How might Jagged1 control the size of the sensory patch in the cochlea during normal development?

As discussed above, a reduction of the sensory patch in the cochlea of *Jagged1* conditional knockout mice might occur because Jagged1 is required only for maintenance of the patch (so that it has degenerated or failed to differentiate in the absence of Jagged1) or because Jagged1 is required for enlarging a small "starter" patch, or perhaps both reasons. Here I discuss these possibilities.

One possible mechanism of enlargement is that Jagged1 positively regulates proliferation in the developing prosensory patch. This seems unlikely as $p27^{Kip1}$, a negative regulator of proliferation, is positively regulated by Jagged1, though this idea cannot be ruled out. It may also be that Jagged1 acts at a distance to specify a broad domain of cells outside its narrow stripe of expression in the cochlea. Another, more attractive, possibility, is that Jagged1 is required to propagate prosensory fate through a positive feedback loop with Notch. Jagged1 activation of Notch on a neighbouring cell stimulates expression of Jagged1 in that cell. In this way, prosensory identity would be spread from cell to cell within a population expressing Notch. In support of this idea, *Jagged1* expression appears to spread in the developing cochlea.

However, this spread of Jagged1 occurs as cells begin to differentiate, several days after the expression of *Math1* is observed. Its possible that the impression of a spreading of Jagged1 distribution is misleading, and that low (but functionally significant) levels of Jagged1 are present in a broader domain from the outset. Alternatively, the spreading pattern of Jagged1 could result from increased Notch activation delivered by nascent hair cells, not simply from auto-induction of Jagged1. Jagged1 may not act to specify a prosensory population, but to facilitate differentiation of hair cells/supporting cells. This issue, of whether Jagged1 is required late, to facilitate differentiation of the patch, or early, for specifying the initial prosensory domain, remains to be resolved.

Chapter 5

The Role of *Delta1* in Inner Ear Development

5.1 Introduction

Delta1 (*Dll1*) is expressed in the developing hair cells of the inner ear sensory epithelium. This pattern of expression supports the idea that hair cells are selected through a process of lateral inhibition, where hair cells escape lateral inhibition mediated by Notch signalling from their neighbours, upregulate their expression of *Delta1*, and inhibit their neighbours from becoming hair cells. This is a simple model, and it may not be entirely correct. While *Delta1* and its orthologues are expressed in the developing hair cells of several model organisms, experiments disrupting its function in chick and in zebrafish have produced results that are not easily interpreted. The role of *Delta1* in the development of hair cells in the mouse inner ear has not been investigated.

Experiments disrupting Notch signalling in zebrafish have yielded results that support the simple lateral inhibition model. In the zebrafish mutant, *mindbomb* (*mib*), a failure of Notch signalling results in all cells of the sensory patch becoming hair cells (Haddon, Jiang et al. 1998; Haddon, Mowbray et al. 1999). In support of the idea that Delta proteins deliver inhibitory Notch signalling is required for correct patterning of zebrafish ear, it is found that a dominant negative form of one of the zebrafish Delta genes, *deltaA*^{dx2}, causes an increase in hair cell number at the expense of supporting cells (Riley, Chiang et al. 1999).

This disruption of hair cell versus supporting cell fate decisions has not been repeated in experiments manipulating Delta function in other vertebrates. Overexpression of *Delta1* in the developing chick inner ear was achieved using replication competent RCAS virus to drive expression of *Delta1* (Eddison, Le Roux et al. 2000). This would be predicted to result in a loss of hair cells caused by increased levels of inhibitory Notch activation. However, counting of hair cells in RCAS-*Delta1* infected regions of the sensory epithelium revealed no change in hair cell numbers. This indicates that *Delta1* is not solely responsible for patterning hair cells and supporting cells.

Expression of *Delta1* in the mouse inner ear is weak and transient, and is therefore difficult to detect using in situ hybridisation, and a good antibody against mouse Delta1 is yet to be found. Therefore the pattern of *Delta1* expression has been studied using mice in which LacZ is expressed under the control of the *Delta1* promoter. These reporter mice show that *Delta1* is expressed in the inner hair cells of the cochlea at E15.5 (Morrison, Hodgetts et al. 1999). A day later, staining is also seen in the outer hair cells. This reflects the difference in timing of inner versus outer hair cell production. In the vestibular system, LacZ staining is detected in all of the sensory patches, with staining first being detected in the developing cristae at E12.5. By E14.5 the stain is clearly seen in the hair cells of all vestibular patches (Morrison, Hodgetts et al. 1999).

As the *Delta1:LacZ* allele is a knockout allele, the expression pattern produced by the *Delta1* promoter was studied in heterozygotes No obvious disruption of hair cell production was seen in these mice which had lost one copy of *Delta1*, though a detailed analysis was not conducted. Mice homozygous for a knockout mutation of *Delta1* die at E10.5, preceding hair cell patterning in the ear (Hrabe de Angelis, McIntyre et al. 1997).

In this chapter I describe the effect of loss of *Delta1* upon development of the sensory patches in the inner ear. It should be emphasised at the outset that *Delta1* is not the only Notch ligand expressed in the nascent hair cells: they express *Jagged2* also, and a knockout mutation of *Jagged2* has effects on the hair cell pattern (Lanford, Lan et al. 1999; Lanford, Shailam et al. 2000). Hair cells are produced in mild excess in the cochlea of *Jagged2* homozygous knockout mice, forming an extra row of outer hair cells and extra inner hair cells in some areas. Thus one of my goals was to see whether the effects of loss of *Delta1* were quantitatively similar or not.

The approach used to study the role of Delta1 in the developing inner ear was similar to that already described for generation of *Jagged1* conditional mice in the previous chapter. Mice carrying a conditional allele of *Delta1* were crossed with mice expressing Cre-recombinase under the control of the *Foxg1* promoter. The resulting conditional knockout mice have a tissue-specific loss of *Delta1* early in development, which allows observation of the homozygous *Delta1* knockout phenotype in the ear, up to late embryonic stages.

5.1.1 Design and production of *Delta1*^{flox} transgenic mice

Mice with a conditional allele of *Delta1*, *Delta1*^{flox}, were provided by Katsuto Hozumi and Michael Owen. A description of the production and analysis of these mice has been published (Hozumi, Negishi et al. 2004). The *Delta1*^{flox} allele LoxP sites lie either side of exons 3 and 4. A Neomycin resistance cassette, flanked by LoxP sites, was inserted into intronic sequence downstream from exon 4. A third LoxP site was inserted in intronic sequence upstream from exon 3. After removal of the neomycin resistance coding sequence, homologous recombinant ES cells containing the *Delta1*^{flox} allele were identified using Southern blot analysis, and were used to produce chimeric mice which served as founders of a *Delta1*^{flox} colony (Figure 5.1).

Delta1^{flox} mice have been validated by work conducted by Katsuto Hozumi. He detected *in vivo* recombination of the *Delta1*^{flox} allele using PCR on tissue samples from *Delta1*^{flox} mice expressing Cre-recombinase under an interferon-inducible promoter. Recombination of the allele brings exons 2 and 5 into sequence. This causes a frame shift that produces a novel stop codon (Hozumi, Negishi et al. 2004). In this way, almost all of the extracellular domain, and all the intracellular domain of *Delta1*, are lost upon recombination of the *Delta1*^{flox} allele. The recombined allele is referred to as *Delta1*$^{\Delta flox}$.

Mice carrying the *Delta1*^{flox} allele were identified by PCR using primers flanking the 5' LoxP site (5XhoD and 3XhoD) (the sequences of all primers used, and the protocol for genotyping are described in Materials and Methods). The recombined, *Delta1*$^{\Delta flox}$ allele was identified using the same 5' primer (5XhoD), and a 3' primer (3DEcoRV) targeted at sequence downstream from the 3' LoxP site (Figure 5.2).

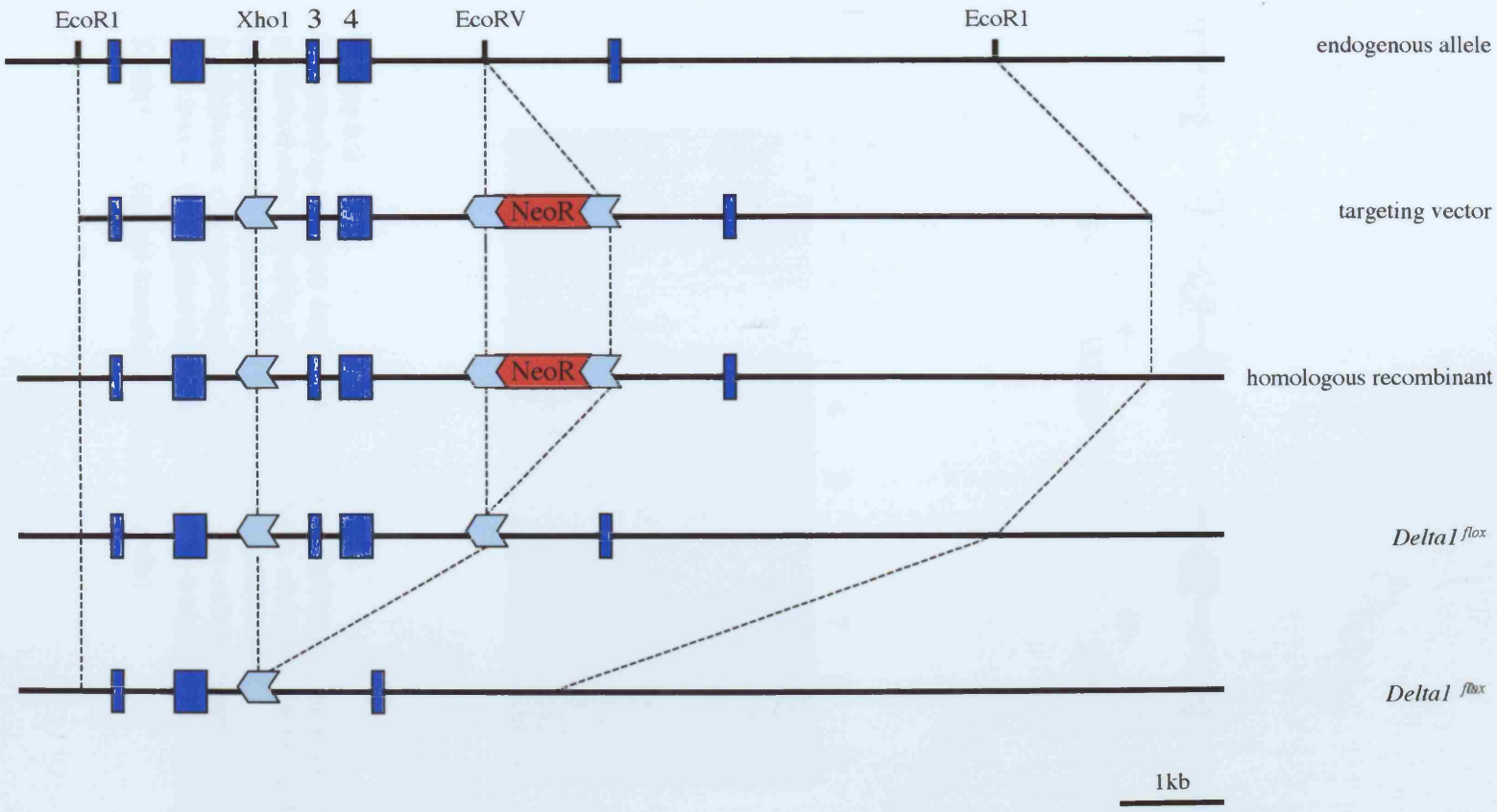


Figure 5.1
Schematic showing the strategy for production of *Delta1* conditional allele.
Exons 3 and 4 (dark blue boxes) were flanked by LoxP sites (light blue arrows) and a Neomycin resistance cassette (red box) was inserted downstream of the targeted sequence.
Partial recombination in vivo was performed to remove NeoR.
(After Hozumi, 2004)

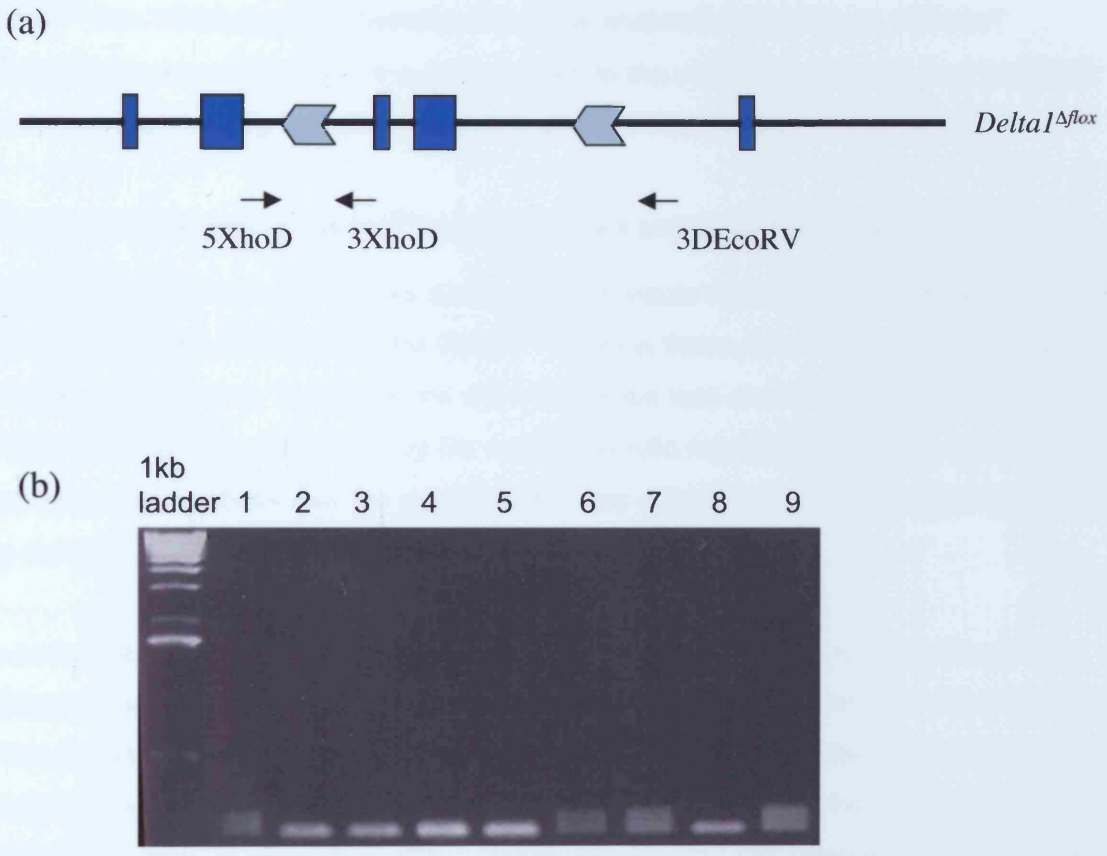


Figure 5.2
Genotyping of litters containing *Delta1* conditional knockout mice.
a) Schematic showing position of primers used for detecting the presence and recombination state of the *Delta1* conditional allele.
b) Example of a genotyping PCR on a gel using primers 5XhoD and 3XhoD on a litter (mice 1-9) containing wildtype (single bands) and heterozygous *Delta1* conditional knockout mice (doublets).

5.2 Results

My method of analysis of the *Delta1* conditional knockout mice is similar to that used for *Jagged1* conditional knockout mice, and the results will be presented in a similar way. For the *Delta1* conditional knockout mice, I start with a brief description of the validation of the *Delta1*^{flox} line. I then go on to describe the gross anatomical phenotype of *Delta1* conditional knockout mice and the defects seen in the vestibular patches, and finally the defect in cell patterning in the sensory epithelium of the cochlea.

5.2.1 Recombination of *Delta1*^{flox} occurs in vivo and produces a knockout allele

Details of the line of *Delta1*^{flox} mice produced by Katsuto Hozumi have now been published, and the validation of the *Delta1*^{flox} allele in these mice has been performed (Hozumi, Negishi et al, 2004). Before this information was available, however, I wanted to confirm that the mice were carrying the correct genetic modification. These controls were also necessary to check that the actual mice in the colony I was maintaining were carrying the correct *Delta1*^{flox} allele in its entirety.

As a check on whether the recombined *Delta1*^{flox} allele, *Delta1*$^{\Delta flox}$, is a functional null, I wanted to examine the phenotype of embryos homozygous for a germline recombination of the *Delta1*^{flox} allele. This could then be compared with the published *Delta1* homozygous knockout phenotype. The knockout phenotype of *Delta1* in mouse has been described in *Delta1:LacZ* mice, in which the coding sequence of *Delta1* has been replaced with that of *LacZ*. Mice homozygous for this knockout allele become haemorrhagic at E10.5 and die at E12 (Hrabe de Angelis, McIntyre et al. 1997). They also exhibit hyperplasia of the CNS, resulting in an enlarged cranium. No line of *Delta1*$^{\Delta flox}$ mice had been produced using fully recombined ES cells, as had been the case with the *Jagged1*^{flox} mice, so I generated mice by recombining the *Delta1*^{flox} allele in vivo. I achieved this by crossing *Delta1*^{flox} females with males expressing Cre recombinase under the control of the *Pgk-1* promoter, *Pgk-Cre*^{m} (Lallemand, Luria et al. 1998) (genotyping of all embryos was performed as described in Materials and Methods). In these mice Cre recombinase is expressed early in the blastocyst, then throughout the embryo. The progeny from these crosses included mice in which recombination of the *Delta1*^{flox} allele would be predicted to have occurred in all tissues.

Delta1$^{\Delta flox}$ heterozygotes generated in this way were then crossed in timed matings to obtain pregnancies containing homozygous *Delta1*$^{\Delta flox}$ embryos. The embryos were collected at E10.5 – E11.5, and were examined under the dissecting microscope. At E10.5, homozygous *Delta1*$^{\Delta flox}$ individuals exhibited an enlarged cranium and widespread haemorrhaging typical of homozygous loss of *Delta1* (Figure 5.3). Homozygous embryos collected a day later, at E11.5, were found dead.

This experiment demonstrates that *Pgk-Cre*^{m} can recombine the *Delta1*^{flox} allele *in vivo*, and that recombination of the allele produces a knockout allele of *Delta1*. To check that *Foxg1-Cre* can also recombine *Delta1*^{flox} in vivo, genomic DNA isolated from mice carrying both *Foxg1-Cre* and *Delta1*^{flox} was used in a PCR that detects *Delta1*$^{\Delta flox}$. *Delta1*$^{\Delta flox}$ was always present in mice carrying both *Delta1*^{flox} and *Foxg1-Cre*.

5.2.2 *Delta1* conditional knockout mice survive to late embryonic stages

It was hoped that *Delta1* conditional knockout mice, having a restricted, tissue specific loss of the gene, would survive to late embryonic stages, allowing observation of hair cell production in the inner ear. I therefore collected litters that were expected to contain *Delta1* conditional knockout mice at E17.5, when hair cells are morphologically identifiable along most of the length of the cochlea.

The breeding strategy for generating *Delta1* conditional knockout mice was similar to that described for the *Jagged1* conditional knockout mice. I wanted to produce mice with two copies of the *Delta1*^{flox} allele (*Delta1*$^{flox/flox}$) and one copy of *Foxg1-Cre* (*Foxg1*$^{Cre/+}$), and suitable control littermates (that is, required wildtype, (*Delta1*$^{flox/flox}$: *Foxg1*$^{+/+}$) or (*Delta1*$^{flox/+}$: *Foxg1*$^{+/+}$) or (*Delta1*$^{+/+}$: *Foxg1*$^{Cre/+}$). Litters predicted to contain embryos of these genotypes were produced by crossing stud male, double heterozygotes (with one copy of *Delta1*^{flox} and one copy of *Foxg1-Cre*), with *Delta1*^{flox} heterozygous females. The resulting litters were predicted to contain homozygous conditional knockout mice (*Delta1*$^{flox/flox}$: *Foxg1*$^{Cre/+}$) at a frequency of one in eight on the basis of Mendelian inheritance.

Twenty-seven litters of mice from parents of these genotypes were collected at E17.5 and genotyped, yielding a total of 214 mice, of which 26 were homozygous conditional

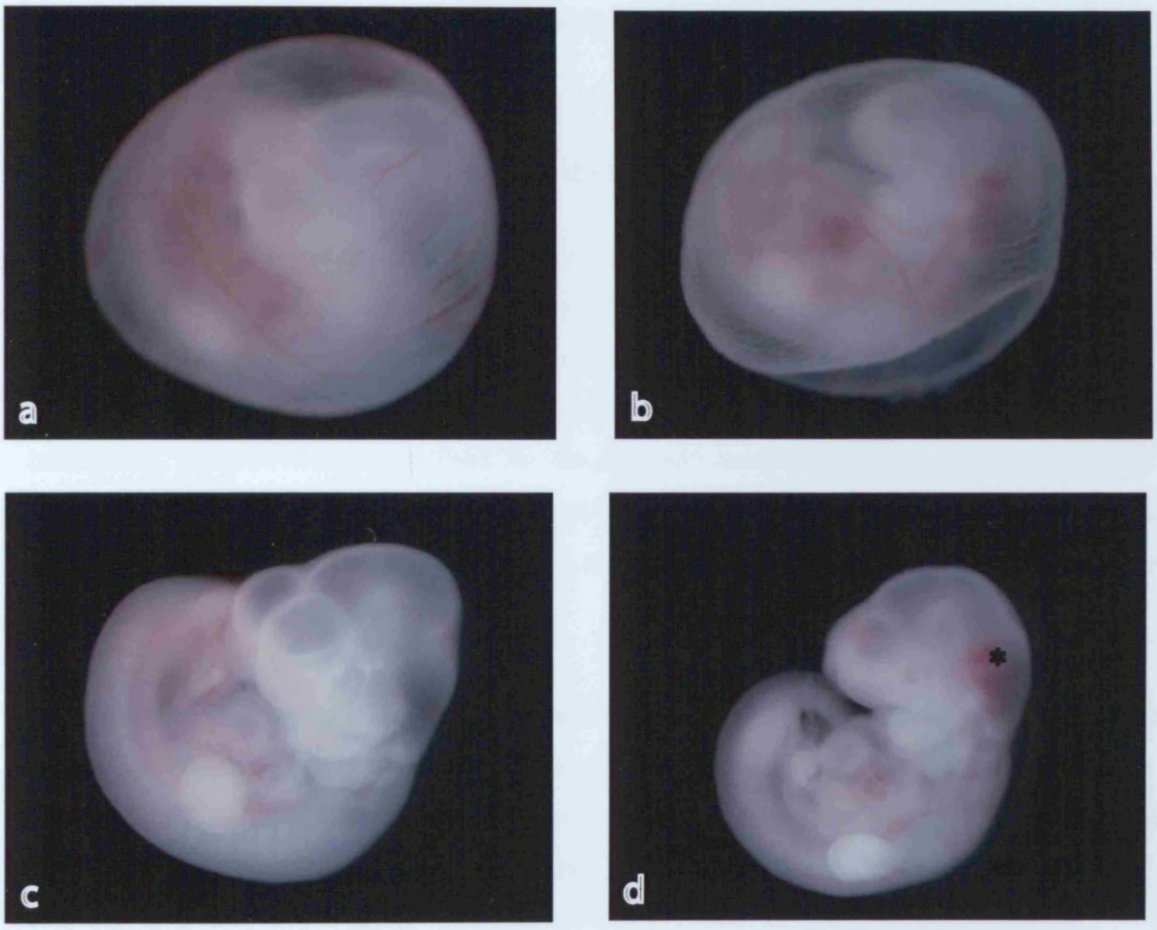


Figure 5.3

Mice homozygous for *Delta1flox* recombined by PGK-Cre exhibit defects similar to those previously described in *Delta1* knockout mice.
Control littermate at E10.5 seen in the yolk sac (a) and out (c), compared to the *Delta1flox* homozygote (b and d).
The *Delta1flox* homozygous embryo appears to have a normal pattern of blood vessels in the yolk sac, but is retarded in development, and exhibits intracranial haemorrhaging (indicated by asterisk) and is becoming nectrotic, though a strong heartbeat was observed in this individual.

knockout mice. The conditional knockout mice were represented at exactly the frequency predicted.

Delta1 conditional knockout mice could be distinguished from their littermates upon collection on the basis of haemorrhaging in the cranium, abnormally shaped heads, and their reduced size compared to control littermates. The brains of these mice appeared severely necrotic. This would be expected given that *Foxg1* is expressed in the brain, and Delta1 has an essential role in neurogenesis (Chitnis, Henrique et al. 1995; Hébert and McConnell 2000).

5.2.3 Loss of *Delta1* causes defects in development of the retina

Having established that the *Delta1*^{flox} allele is recombined by *Foxg1-Cre*, and that it produces a knockout allele of *Delta1*, as a further check I wanted to look at histological effects in a well-characterized tissue where recombination of the conditional allele was expected to occur, and where it would be expected to give predictable defects in tissue patterning. As previously described for the *Jagged1* conditional mice, *Foxg1-Cre* drives expression of Cre-recombinase throughout the lens placode and the nasal half of the optic cup at E9.5 (Hébert and McConnell 2000), and thus recombines conditional alleles in the developing eye.

Mice were collected, paraffin sectioned, and stained with H & E. This revealed defects in retinogenesis in the nasal half of the retina. At E17.5, rosettes of cells are formed in the retina of conditional knockout mice (Figure 5.4). This phenotype is similar to that seen in *Hes1* null mutant mice, where rosettes form as a result of premature neurogenesis (Tomita, Ishibashi et al. 1996).

Thus in the eye at least, recombination of the *Delta1*^{flox} allele mediated by *Foxg1-Cre* appears to occur efficiently and in the expected, restricted pattern.

5.2.4 Loss of *Delta1* causes a mild defect in outgrowth of the cochlea

Superficially, the inner ear of the *Delta1* conditional knockout appears normal upon dissection (Figure 5.5). Closer inspection, however, reveals some abnormalities. During normal development, the cochlea undergoes progressive coiling as it lengthens, so that by E15.5 the cochlea has achieved one and a half turns (Morsli, Choo et al. 1998). Two days

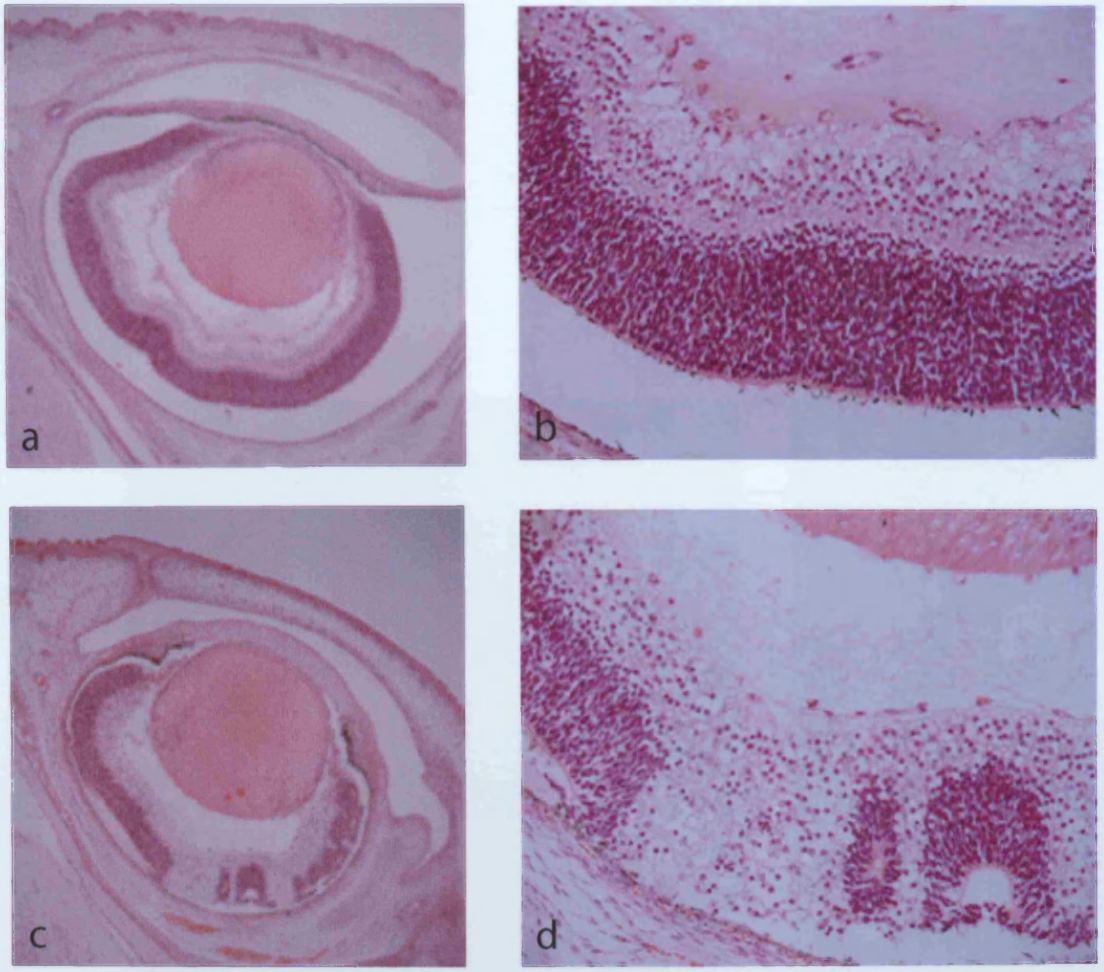


Figure 5.4
H & E staining of the eye at E17.5 in a normal littermate (a,b) and in the *Delta1* conditional knockout (c,d). Rosettes of cells can be seen in the nasal portion of the retina in the *Delta1* conditional knockout mouse, shown in (d).

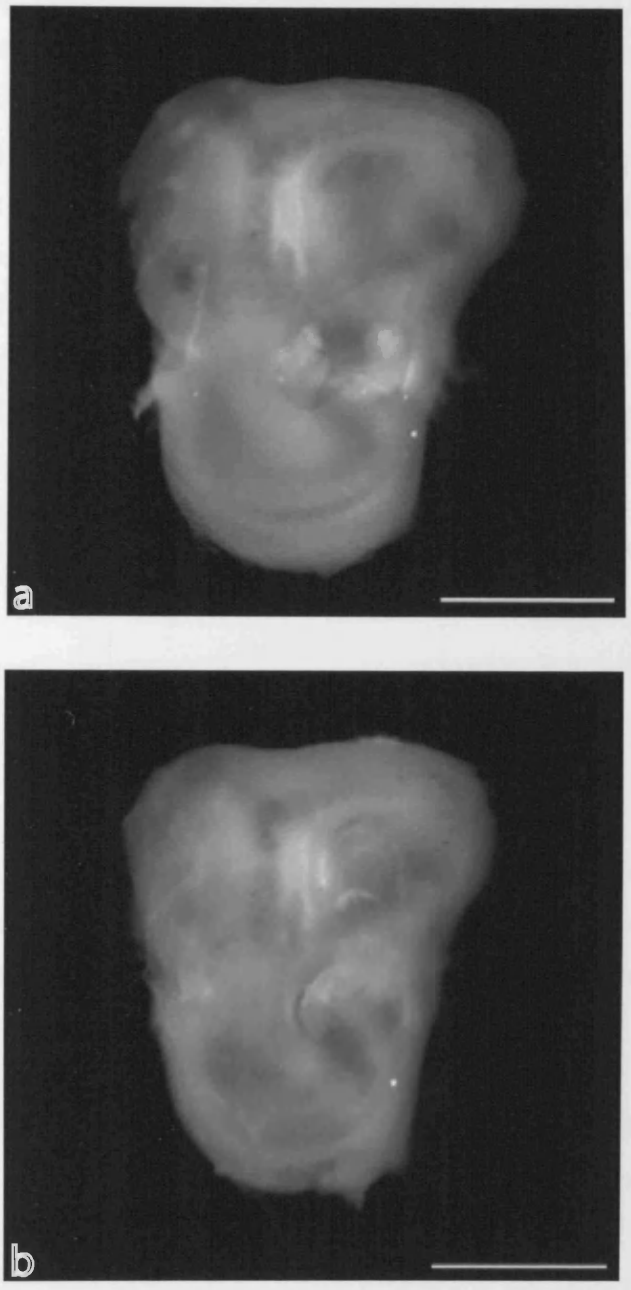


Figure 5.5

Gross morphology of the inner ear in the *Delta1* conditional knockout mouse.

Images of medial views of the inner ear dissected out at E17.5 show that there are no obvious defects in the semicircular canals, as had been seen in the *Jagged1* conditional knockout.
Scale bar is 1mm

later, at E17.5, the cochlea has achieved the mature morphology, having one and three-quarter turns. In the *Delta1* mutant, this growth is slightly delayed or diminished, so that E17.5 *Delta1* conditional knockout mice have not achieved the normal number of turns (Figure 5.6). I measured the length of the cochlea using confocal images of flat mounted portions of the cochlea at E17.5 using Zeiss LSM 5 Image Browser software (as described in Materials and Methods). This analysis revealed that at E17.5, the cochleas from *Delta1* conditional knockout mice were 70% of the length of those from control littermates (Table 5.1). Those from mutant mice were an average length of 3144 ± 78μm (mean±SEM:n = 6) compared to those from littermate controls, which were 4454 ± 56μm (mean±SEM; n = 6). One litter was collected a day later, at E18.5. The length of the cochlea from the *Delta1* conditional knockout from this litter was closer to that of littermate controls, at 92% of the normal length (3935μm compared to 4294μm), suggesting that the reduced length of the cochlea is due to a delay in its growth.

5.2.5 Loss of *Delta1* disrupts development of vestibular sensory patches in a different manner from loss of *Jagged1*

In contrast to the *Jagged1* conditional knockout mice, no obvious defects in the gross morphology of semicircular canals were seen in the *Delta1* conditional knockout mice at E17.5. In order to study the patterning of vestibular sensory patches in the *Delta1* conditional knockout in detail I used frozen sections of the head from E17.5 individuals. I stained sections through the entire inner ear from three *Delta1* conditional knockouts and their littermate controls with the anti-Jagged1 antibody to mark the sensory epithelia. Sections were counterstained with fluorescent phalloidin to visualise hair cells with their upregulated cortical actin and actin-rich hair bundles.

All three cristae were present in the *Delta1* conditional knockout mice, though they appeared reduced in size. The epithelium in each patch appeared to contain the normal pattern of hair cells and supporting cells, staining positive for Jagged1, and having the normal bilayered arrangement of the two cell types. In two of the three *Delta1* conditional knockout mice analysed in this way, the utricular macula was present, but the saccular macula was not. In the third individual, both maculae were absent. I measured the volume of the sensory epithelia in one individual, in which the utricular macula and the three cristae were present, using Zeiss LSM 5 Image Browser software (as described in Materials and Methods). This analysis showed that the total volume of the three cristae and the utricular macula were markedly reduced in the *Delta1* conditional knockout, at

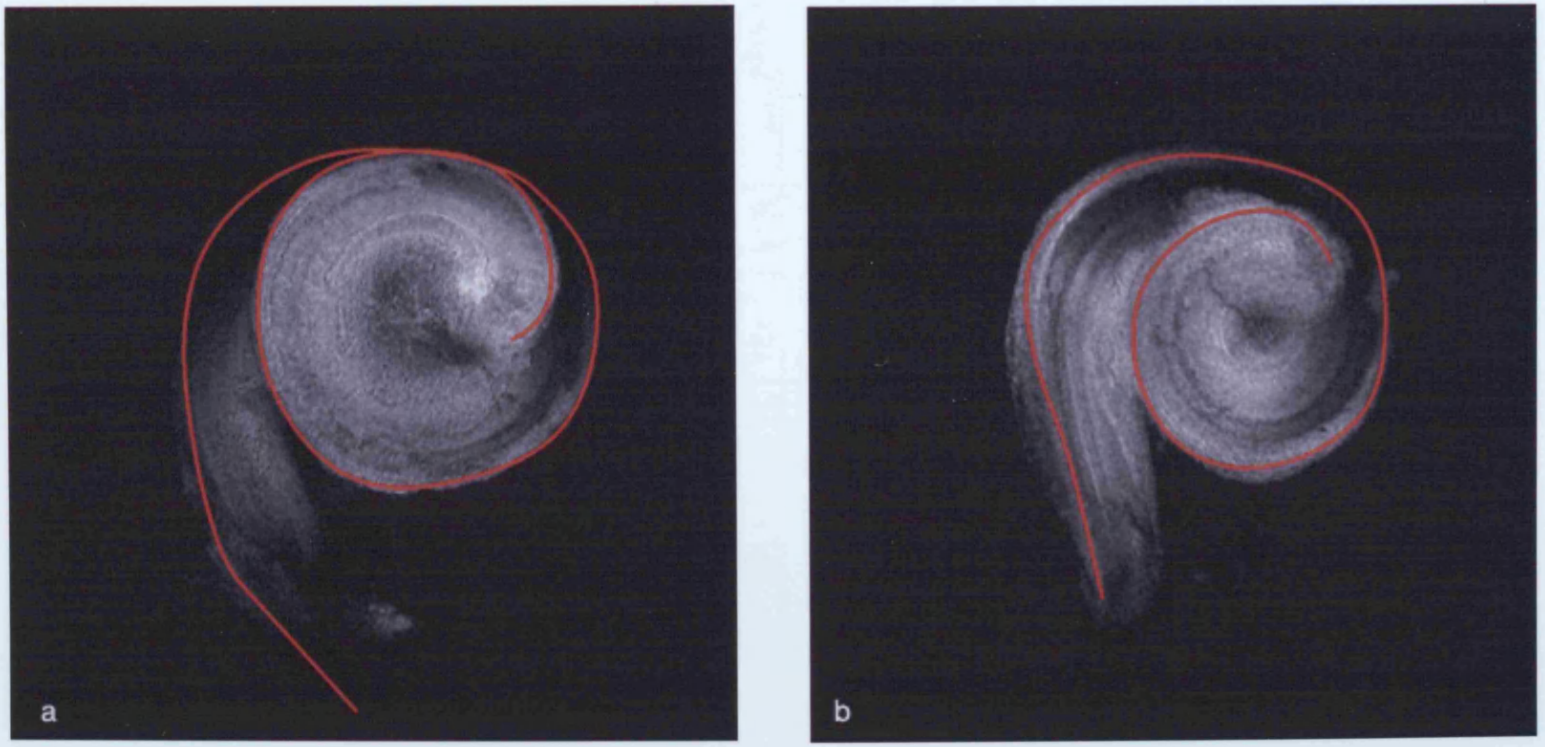


Figure 5.6

The outgrowth and coiling of the cochlea is disrupted by loss of *Delta1*.
Red lines indicate the length of the cochlea measured, in order to compare wildtype (a) and *Delta1* conditional knockout (b) cochleas at E17.5. Cochleas were stained with phalloidin.

Genotype	Stage	No	Litter No.	Apex portion (μm)	Middle portion (μm)	Basal portion (μm)	TOTAL LENGTH
Delta1$^{\Delta flox/\Delta flox}$	E17.5	1a	1	1310	1189	582	3081μm
Delta1$^{\Delta flox/\Delta flox}$	E17.5	1b	1	1299	1132	739	3170μm
Delta1$^{flox/+}$	E17.5	3a	1	2357	1142	872	4371μm
Delta1$^{flox/+}$	E17.5	3b	1	1202	1863	1803	4868μm
Delta1$^{\Delta flox/\Delta flox}$	E17.5	2a	2	1224		2272	3496μm
Delta1$^{\Delta flox/\Delta flox}$	E17.5	2b	2	1245		1708	2954μm
Delta1$^{flox/+}$	E17.5	6a	2	2130		2171	4301μm
Delta1$^{flox/+}$	E17.5	6b	2	2152		2045	4197μm
Delta1$^{\Delta flox/\Delta flox}$	E17.5	3a	2	1330		1824	3154μm
Delta1$^{\Delta flox/\Delta flox}$	E17.5	3b	2	1416		1595	3011μm
Foxg1 $^{Cre/+}$	E17.5	4a	2	1607	1242	1650	4499μm
Foxg1 $^{Cre/+}$	E17.5	4b	2	2494		1996	4490μm
Delta1$^{\Delta flox/\Delta flox}$	E18.5	2a	3	1917	1370	714	4001μm
Delta1$^{\Delta flox/\Delta flox}$	E18.5	2b	3	1347		2521	3868μm
Foxg1 $^{Cre/+}$	E18.5	12a	3	1348	2217	953	4518μm
Foxg1 $^{Cre/+}$	E18.5	12b	3	1440	1628	1002	4070μm

Table 5.1

Length of the cochlea in *Delta1* conditional knockout mice compared to their littermate controls.

7,380,000μm^3, compared to 9,314,370μm^3 in the littermate control (Figure 5.7). This reduction in the size of the sensory epithelia may well reflect the fact that *Delta1* conditional knockout mice were generally smaller than their normal littermates. Severe reduction of the utricular or saccular macula was correlated with reduction of the utricle or saccule as a whole.

5.2.6 Hair cell differentiation occurs early and in excess in the *Delta1* conditional knockout cochlea

If *Delta1* is indeed required to control hair cell production in the sensory patches by lateral inhibition, one might expect to see not only an excessive production of hair cells upon its removal, but also an early production of hair cells, as seen in the zebrafish mutant, *mindbomb*.

To investigate whether this is the case, I collected litters containing *Delta1* conditional knockout individuals at E17.5 (and genotyped each embryo, as described in Materials and Methods) and dissected the inner ear to expose the sensory epithelium of the cochlea. I then stained the cochleas as wholemounts using fluorescent phalloidin to visualise developing hair cells.

In the normal cochlea hair cell differentiation occurs in a wave that begins in the mid-basal part and travels in a wave that moves basally and apically (Lim and Anniko 1985). This process begins at E15.5, and reaches the apex at early postnatal stages. Thus, in the wildtype cochlea at E17.5 no differentiated hair cells can be seen in the apex, and an upregulation of cortical actin in these cells that is seen at the start of their differentiation is not yet apparent. In contrast, hair cell differentiation has occurred prematurely in the apex of *Delta1* conditional knockout cochleas. In addition, hair cells in this region have been produced in huge excess (Figure 5.8). Between 3 and 5 extra rows of outer hair cells are formed, and the inner hair cell row appears to have been duplicated.

At E17.5 hair cells in the middle part of the normal cochlea have differentiated and the stereotyped pattern of three rows of outer hair cells and one row of inner hair cells is established. Hair cells are immature, but can be easily identified on the basis of the accumulation of actin at the plasma membrane and their developing hair bundles. In the middle part of the *Delta1* conditional knockout cochlea the overproduction of hair cells is

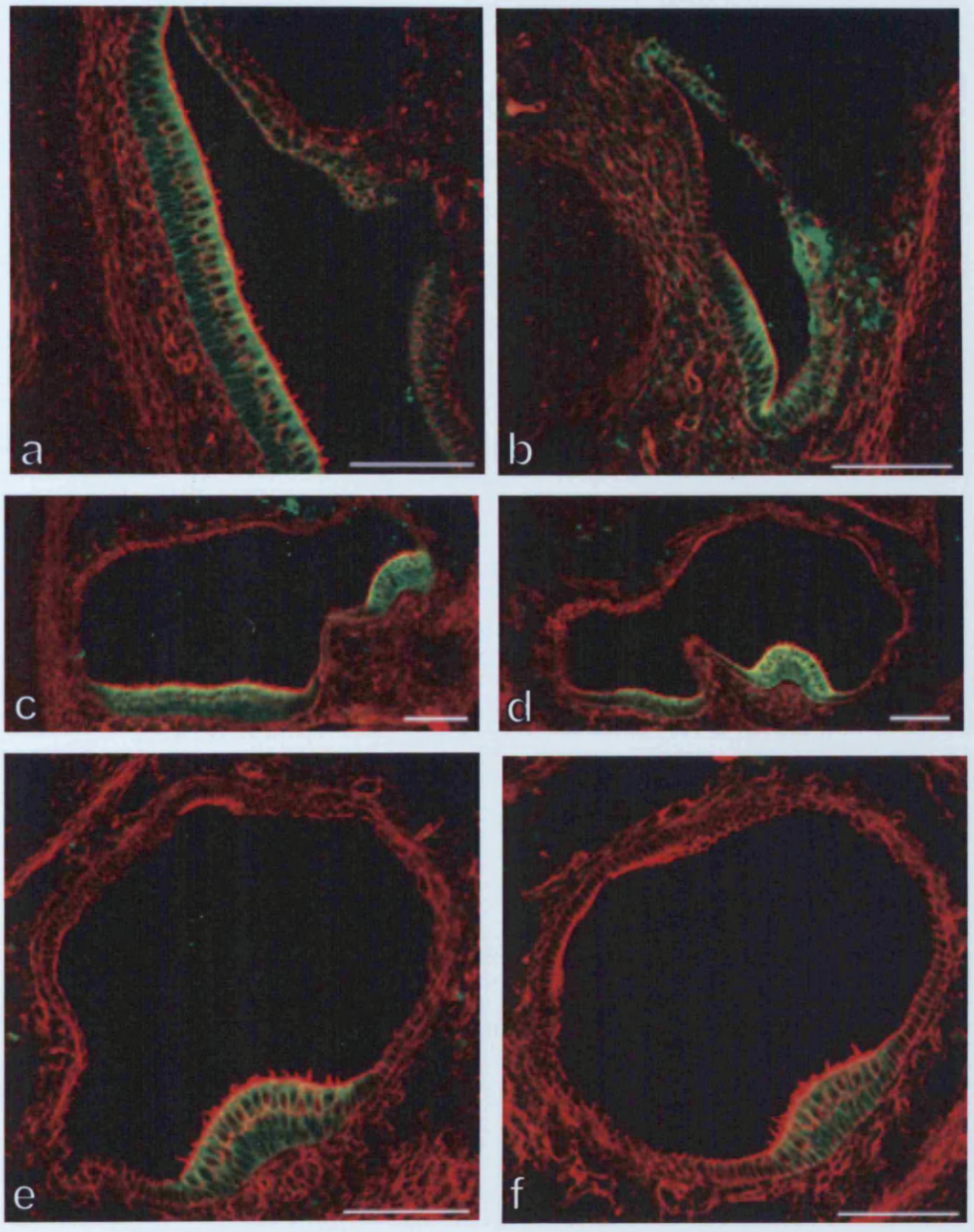


Figure 5.7
Vestibular defects in the *Delta1* conditional knockout at E17.5.
Sections were stained with Jagged1 antibody (green) and phalloidin (red).
The saccule and its macula were either dramatically reduced (compare control in a to mutant in b) or lost in the *Delta1* conditional knockout. Likewise, the utricle and its macula were either reduced (compare control in c to mutant in d) or missing. The cristae appeared smaller (compare control in e to mutant in f), but were otherwise patterned normally.
Scale bars are 100μm.

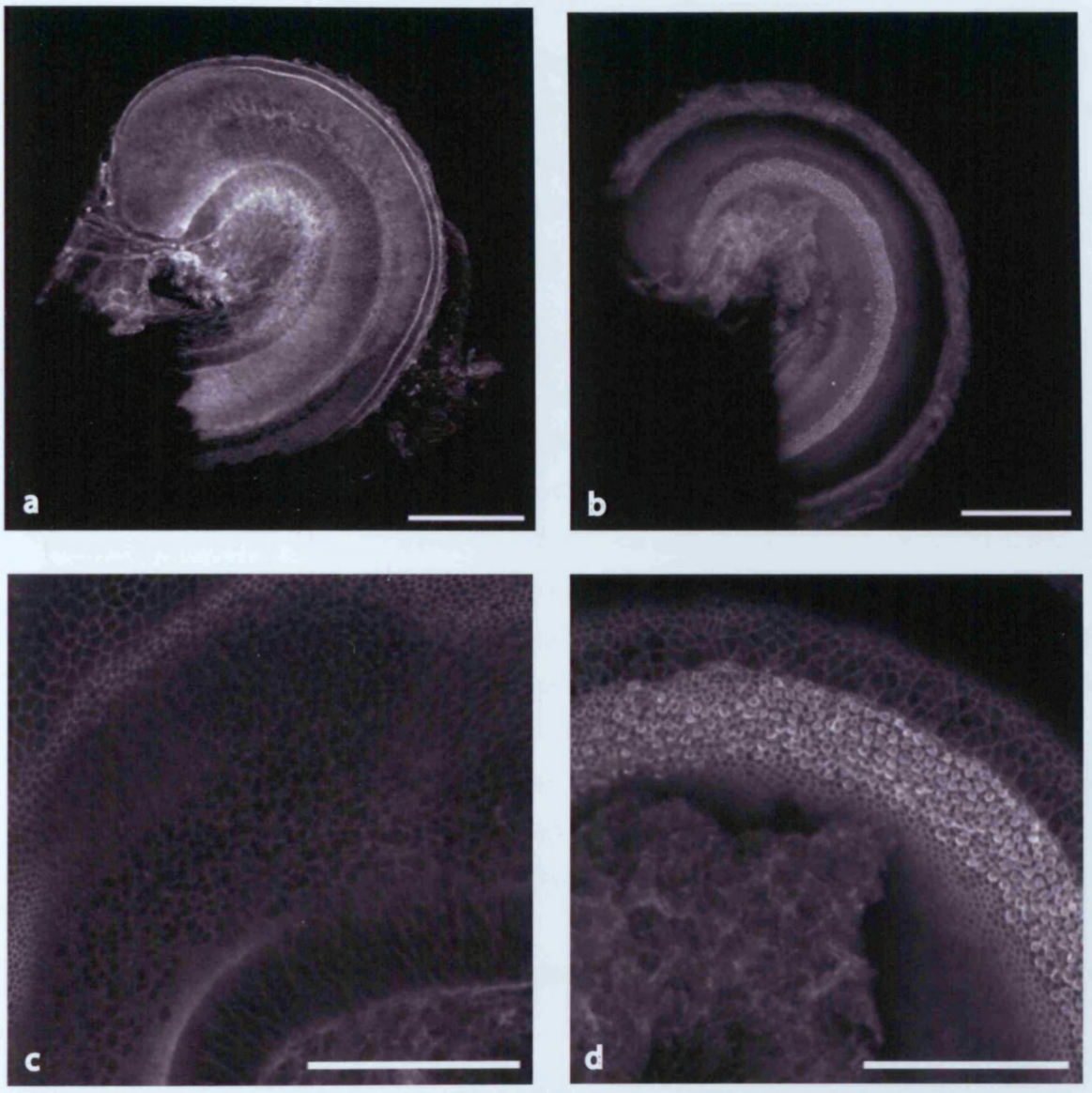


Figure 5.8

Phalloidin stain of the cochlea apex from E17.5 littermate control mouse (a) and a *Delta1* conditional knockout mouse (b).
Close up images reveal numerous extra rows of hair cells have formed prematurely in the *Delta1* conditional knockout (d) compared to this region in littermate controls (c)
Scale bars are 200µm (a,b) and 100µm (c,d).

again obvious, though less extreme than in the apex. An extra row of outer hair cells has been produced, and the number of inner hair cells has also increased (Figure 5.9). The arrangement of supernumary inner and outer hair cells differs, in that inner hair cells are sometimes seen to be in contact with one another.

A similar contrast between mutant and wildtype is seen in the basal part of the cochlea at this stage. In littermate controls, the pattern of four rows of hair cells extends to the base of the cochlea. In *Delta1* conditional knockouts, the pattern of hair cells in this region is altered in the same way as that seen in the middle portion: an extra row of outer hair cells, and an increased number of inner hair cells.

A more subtle increase in the number of hair cells was also seen in *Delta1* conditional knockout heterozygotes, that is, mice with one copy of *Delta1*^{flox}, one copy of the normal *Delta1* gene, and a copy of *Foxg1-Cre*. I collected three such heterozygotes and control littermates at E17.5, and stained them with fluorescent phalloidin. I photographed a 1500μm length of the sensory patch from the basal end of the cochlea using the confocal microscope, and counted hair cells within this area of the cochlea. An extra row of outer hair cells and extra inner hair cells were seen in some portions of this length of cochlea in the *Delta1* conditional knockout heterozygotes (Figure 5.10). Such supernumary hair cells were also seen in control cochleas, though not as frequently. These ectopic hair cells slightly increased the average number of hair cells seen in the sampled 1500μm length of the sensory epithelium from 1495 ± 43 (mean±SEM;n=3) hair cells, as seen in the controls, to 1560 ± 21 (mean±SEM;n=3) (Table 5.2).

5.2.7 The excessive production of hair cells is not accompanied by any reduction in the number of supporting cells

The lateral inhibition hypothesis would predict that in the absence of *Delta1*, less or no inhibitory signal would be sent from developing hair cells, and more cells within the patch would escape inhibition via the Notch pathway, and develop as hair cells. One would then expect to see a decrease in the number of supporting cells in the sensory patch balancing the increase in hair cells. To determine whether this is the case in the *Delta1* conditional knockout cochlea, wholemount E17.5 cochlea preparations were immunostained with a Jagged1 antibody to mark supporting cells. In the normal cochlea Jagged1 is localised to the membranes of supporting cells, where it can be seen in the apical projections of these

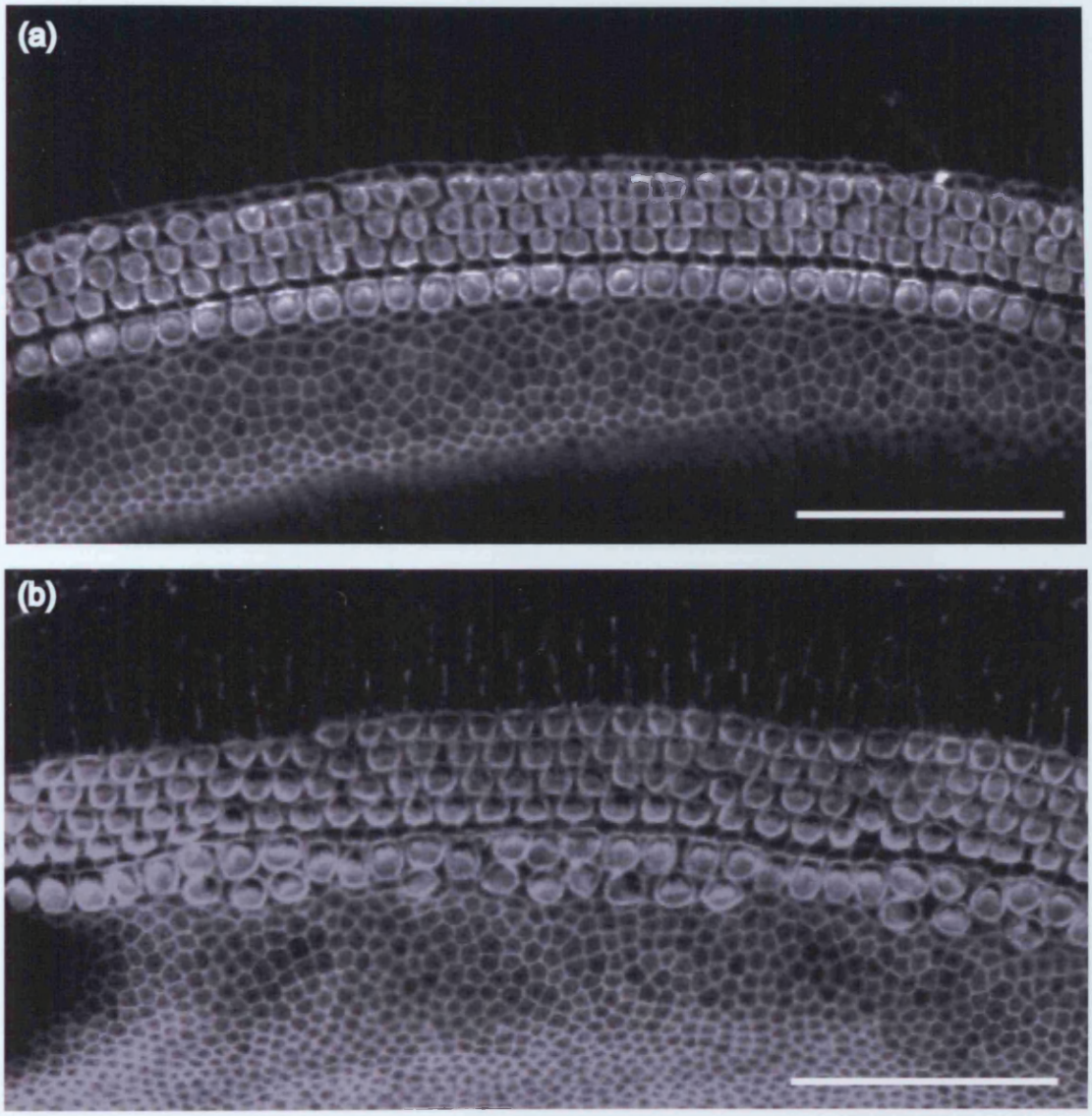


Figure 5.9
Hair cell production in the *Delta1* conditional knockout; middle/basal cochlea.
Phalloidin stain of the cochlea in wholemount at E17.5, showing the normal pattern of hair cells in the control mouse (a), and the excess of hair cells seen in the middle part of the *Delta1* conditional knockout (b). The same defect in hair cell production was seen in the basal part of the mutant cochlea.
Scale bars are 50μm.

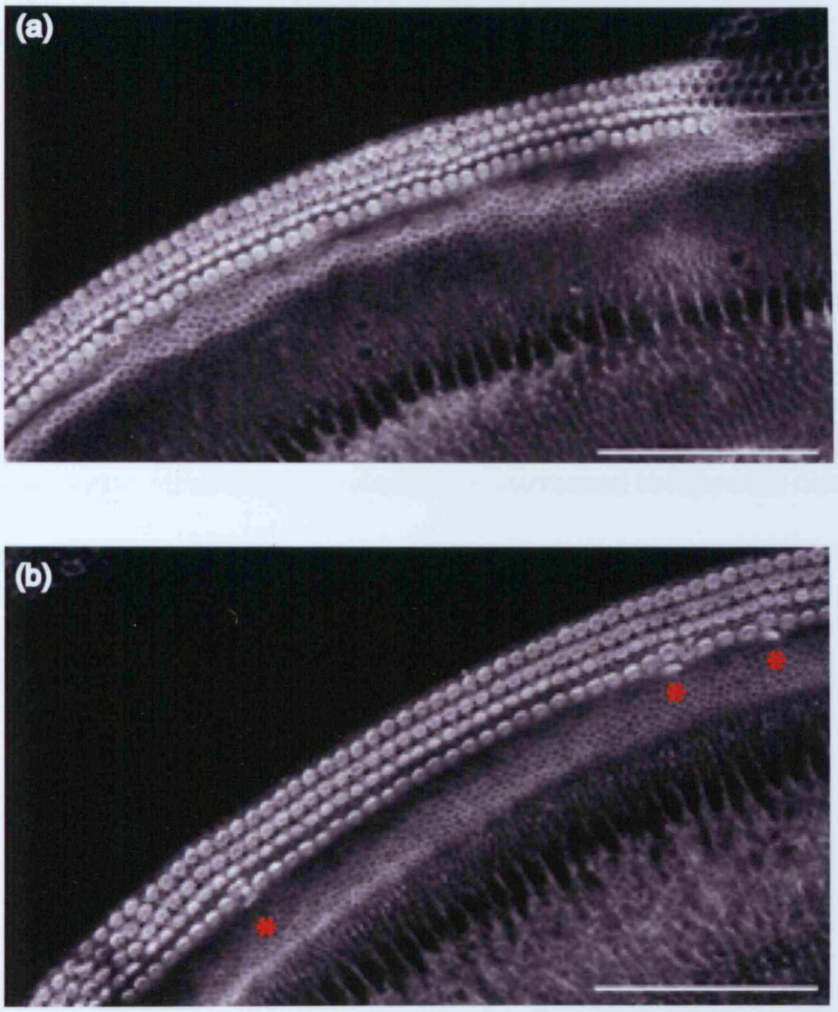


Figure 5.10

The pattern of hair cells is visualised by staining of the cochlea with phalloidin in wholemount from wildtype (a) and heterozygous *Delta1* conditional knockout (b) mice.
Red asterisks in (b) indicate an area where there are four rows of outer hair cells instead of the normal three rows (left), and extra inner hair cells (right).
Scale bars are 100µm.

Table 5.2

Hair cell counts in heterozygous *Delta1* conditional knockout cochleas at E17.5.

Genotype	Extra OHCs	Extra IHCs
Foxg1$^{Cre/+}$ *:Delta1*$^{\Delta flox/+}$	45	5
wildtype	2	1
Foxg1$^{Cre/+}$ *:Delta1*$^{\Delta flox/+}$	7	2
Foxg1$^{Cre/+}$	0	1
Foxg1$^{Cre/+}$ *:Delta1*$^{\Delta flox/+}$	22	1
wildtype	9	0

Counts of extra inner and outer hair cells in the basal 1.5mm of the cochlea from heterozygous *Delta1* conditional knockout mice, compared to littermate controls.

Mean number of extra hair cells in heterozygous *Delta1* conditional knockout mice was 2.6, compared to 0.7 in control mice.

Mean number of extra outer hair cells in heterozygous *Delta1* conditional knockout mice was 24.7, compared to 3.7 in the control mice.

cells surrounding the hair cells at the surface and in the superficial part of the sensory epithelium, and surrounding the supporting cell body in the basal part of the epithelium. At E17.5 Jagged1 stains all supporting cells of the sensory epithelium and some cells in the greater epithelial ridge (i.e. medial to the inner hair cells). In the wildtype animal this includes the inner border cells (which are medial to the inner hair cells), the inner and outer pillar cells (which separate inner and outer hair cells), and three rows of Deiters' cells (which are associated with the three rows of outer hair cells). Jagged1 is absent from the Hensens' cells, which lie at the lateral border of the organ of Corti, and from the numerous Claudius cells lying beyond them.

The pattern of Jagged1 staining in the organ of Corti of *Delta1* conditional knockout cochleas does not differ from that seen in the normal cochlea. Jagged1 staining is visible at the membranes of supporting cells, as in the wildtype cochlea, and the population does not seem to be significantly reduced. To determine whether there might nevertheless be a subtle change in the ratio of hair cells and supporting cells, I counted hair cells and Jagged1-positive supporting cells in *Delta1* conditional knockouts and in their wildtype littermates.

Cell counts were restricted to cells of the sensory patch that lay outside the row of pillar cells. I limited my analysis to this outer region, because the borders of *Jagged1* expression in the inner region are only hazily defined. This makes counting of Jagged1 positive cells in this inner region difficult to standardise. By contrast, the lateral margin of Jagged1 staining, in the last row of Deiters' cells, is sharp and clearly defined. The count in this study was thus restricted to the outer hair cells and their associated supporting cells: the outer pillar cells and the Deiters' cells. Using wholemount cochleas stained with Jagged1 antibody, phalloidin and DAPI, I produced z-stacks of confocal images which spanned the depth of the epithelium, going from the hair bundles at the apices of hair cells to the spiral artery that runs below the basement membrane on which the sensory epithelium rests (Figure 5.11). The numbers of hair cells (identified by their apical nuclei and hair bundles) and of supporting cells (identified by their positive staining for Jagged1 and their position in the sensory epithelium) were then counted per 100μm length of sensory patch in the middle turn of the cochlea conditional knockout mice and their littermate controls. 7 conditional knockout individuals and 6 littermate controls were analysed in this way.

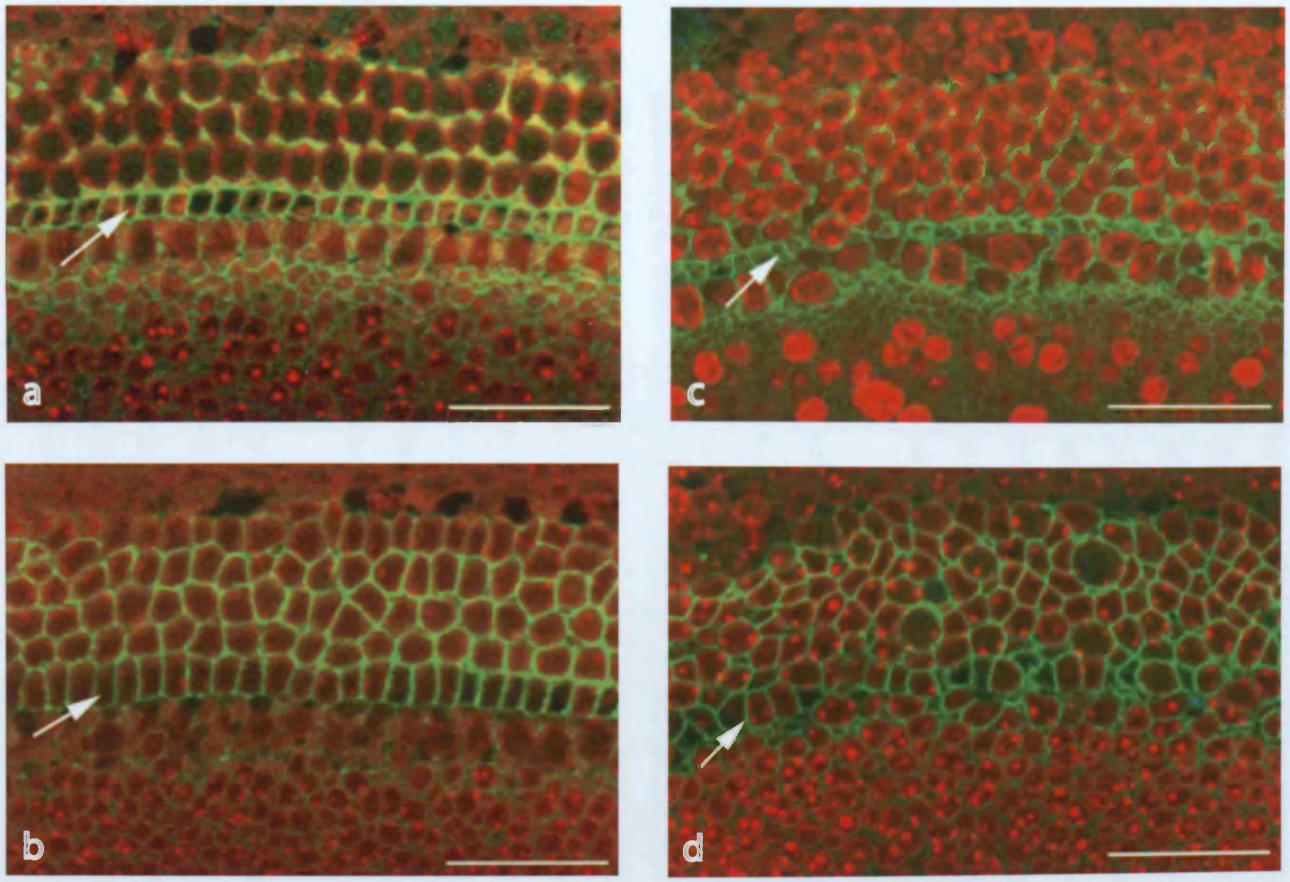


Figure 5.11
Confocal images of the sensory patch in the middle part of the cochlea at E17.5. Jagged1 (green) is seen in the supporting cells in both the littermate control (a,b) and the *Delta1* conditional knockout (c,d). It can be seen in the apical processes of the supporting cells at a superficial level in the epithelium (a and c), at the level of the hair cell nuclei, and at a basal level, at the level of the supporting cell nuclei (b and d). Arrows indicate the location of the inner pillar cell row. Scale bars are 50μm.

These counts revealed that an increase in supporting cells accompanies the increase in hair cells seen in the *Delta1* mutant (Table 5.3) (Figure 5.12). In both the *Delta1* conditional knockout and the control cochlea, there were approximately 15 hair cells per row in the 100μm length sample area. This gave an average of 62 ± 5.1 outer hair cells (mean±SEM;n=7) in the *Delta1* conditional knockout, comprising four orderly rows, and 46 ±1.7 (mean±SEM;n=6) in control mice, comprising the normal three rows. The number of outer hair cells thus increased in the *Delta1* conditional knockout by approximately 35%. The number of Deiters' cells was also increased in the mutant cochlea. Counting of this cell type revealed a 14% increase in the *Delta1* conditional knockout compared to littermate controls (*Delta1* conditional knockout 80 ± 7.4; controls 70 ±4.8). In the normal situation, four to five rows of supporting cells can be seen, whereas in the mutant cochlea, five to six rows are observed.

The increase in supporting cell numbers appears to be due to the formation of an extra row of Deiters' cells. This is clearly illustrated in one *Delta1* conditional knockout individual that showed an exception to the general rule, in that it contained an area with the normal three rows of outer hair cells adjacent to an area with the four rows characteristic of such mutants. This combination of normal-looking and disturbed patterns was not seen in any of the other *Delta1* conditional knockouts studied, and is perhaps due to a failure of the $Delta1^{flox}$ allele to be recombined throughout the cochlea. However, it provided a useful check on my conclusions from the other specimens. A z-series taken at the meeting point of these two differently patterned regions clearly shows an extra row of supporting cells accompanying the extra row of outer hair cells (Figure 5.13).

This increase in the number of outer hair cells and Deiters' cells does not appear to occur at the expense of immediately adjacent supporting cell types. Pillar cells, which can clearly be seen in each case, were present in the same numbers in both mutant and control sensory epithelia. Counts of Hensens cells, using confocal images of the surface of the epithelium in wholemount cochleas stained with phalloidin, revealed that the number of this cell type does not change between the *Delta1* conditional knockout organ of Corti and that of the littermate controls, with both genotypes having approximately 25 Hensens cells per 100μm.

The strongest evidence that supporting cells as well as hair cells are produced in excess in the absence of *Delta1* comes from analysis of the sensory epithelium in the apex of the

Table 5.3

Hair cell and supporting cell counts for the middle portion of the cochlea at E17.5 in Delta1 conditional knockout mice compared to littermate controls.

Litter numbers are inserted here only to identify littermates and do not relate to other experiments.

Genotype	Litter No.	No. Hcs per. 100um	No. Scs per.100um	SC PER. HC
Foxg1$^{Cre/+}$:Delta1$^{\Delta flox/+}$	Litter 1	61	73	1.2
wildtype	Litter 1	46	66	1.4
Foxg1$^{Cre/+}$:Delta1$^{\Delta flox/+}$	Litter 1	64	76	1.2
Foxg1$^{Cre/+}$	Litter 2	47	76	1.6
Foxg1$^{Cre/+}$:Delta1$^{\Delta flox/+}$	Litter 2	71	96	1.4
wildtype	Litter 3	46	66	1.4
Foxg1$^{Cre/+}$:Delta1$^{\Delta flox/+}$	Litter 3	65	77	1.2
wildtype	Litter 4	45	67	1.5
Foxg1$^{Cre/+}$:Delta1$^{\Delta flox/+}$	Litter 4	56	79	1.4
wildtype	Litter 5	49	76	1.6
Foxg1$^{Cre/+}$:Delta1$^{\Delta flox/+}$	Litter 5	59	80	1.4
wildtype	Litter 5	44	70	1.6
Foxg1$^{Cre/+}$:Delta1$^{\Delta flox/+}$	Litter 5	58	79	1.4

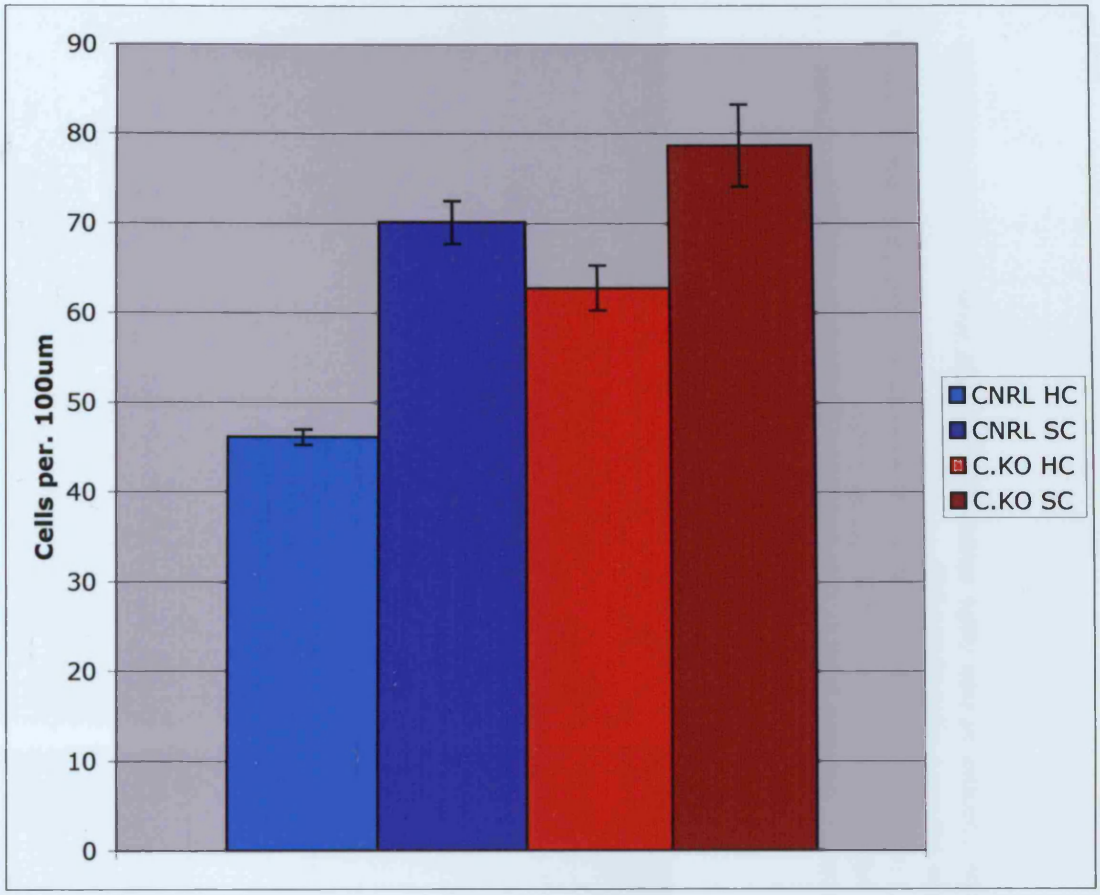


Figure 5.12

Graph showing the number of hair cells and supporting cells per 100µm length of the middle part of the cochlea in littermate controls (blue) and in *Delta1* conditional knockout mice.

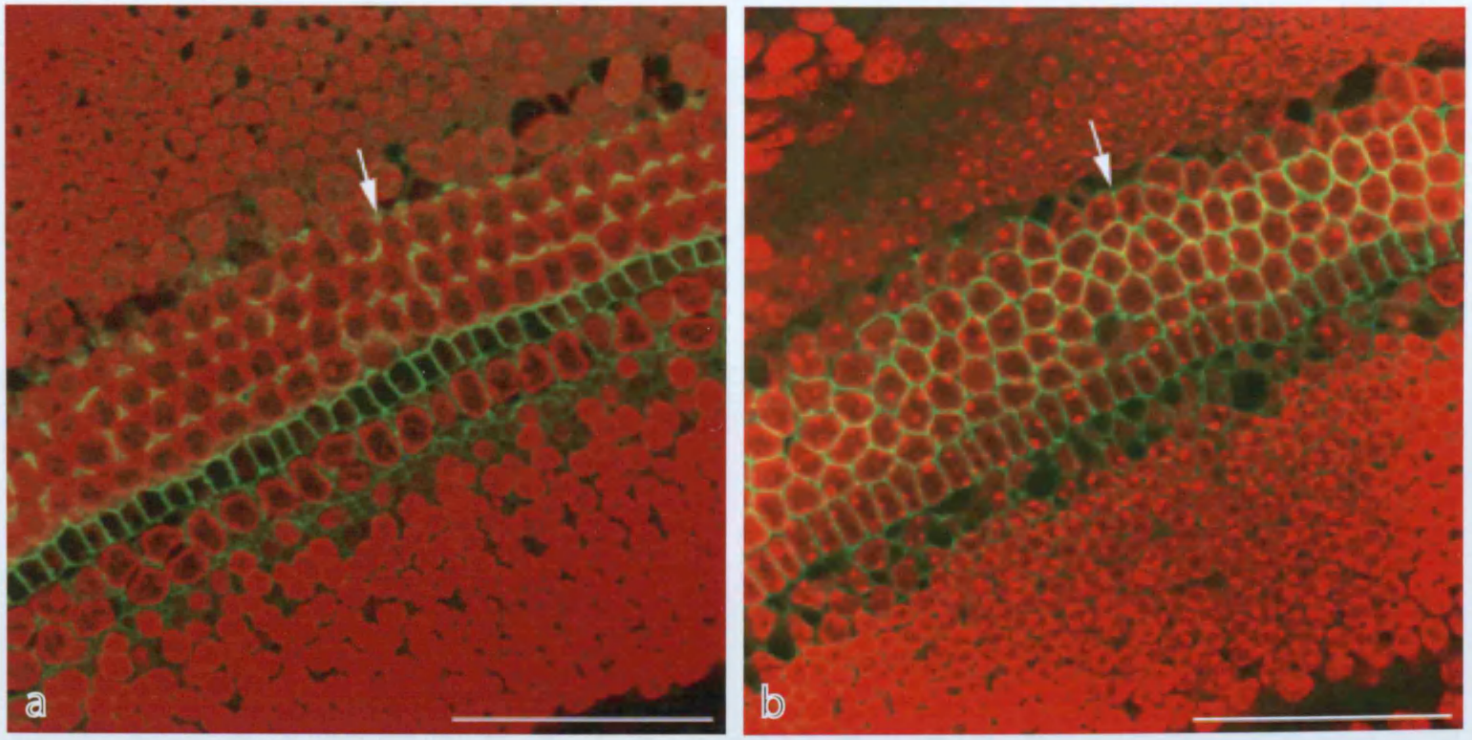


Figure 5.13
Images show the sensory epithelium in the cochlea at E17.5 in a *Delta1* conditional knockout mouse. Wholemount cochleas were stained with Jagged1 (green) and DAPI (red).
At a superficial level of the epithelium (a) the optical section is at the level of the hair cell nuclei. The nuclei of the supporting cells lie deeper in the sensory epithelium (b).
The arrow marks the point at which the number of hair cells changes,from four rows of outer hair cells, to three rows. Scale bar is 50μm.

cochlea. In this area of the cochlea, the Jagged1 antibody staining encompasses the abnormally broad sensory patch, suggesting that supporting cells are produced in the normal ratio, even where there is a gross excess of hair cells (Figure 5.14). Cell counts in the apex at E17.5 revealed a greater increase in both hair cells and supporting cells per 100μm length of the epithelium compared to the middle portion of the cochlea (Table 5.4). There is no littermate control available for these counts, as hair cell production has not occurred in the apex of normal cochleas at this stage. In the apical region of the *Delta1* conditional knockout, however, there were approximately two rows of inner hair cells and a huge excess of outer hair cells. While the rows are rather disorganised, approximately 6-8 rows of outer hair cells can be identified, compared to the normal three rows that will eventually form in the apex of the wildtype cochlea. The arrangement of hair cells and supporting cells is similar to that seen in middle regions of the cochlea, both in the wildtype and the Delta1 conditional knockout, and the ratio of supporting cells to hair cells is higher – quite the opposite of expectations of the simple lateral inhibition hypothesis.

5.2.8 Proliferation in the sensory patch is not dramatically deregulated in the *Delta1* conditional knockout cochlea

The results I have described so far indicate that *Delta1* has a role in determining the size of the sensory patch - a role not previously ascribed to this particular Notch ligand. What causes the increase in the size of the patch in the conditional knockout cochlea? The loss of *Delta1* could increase the size of the initial prosensory patch. Alternatively, loss of *Delta1* could result in loss of cell death in the developing patch, or in increased proliferation within the patch, or abnormal recruitment into the patch at a later stage. An effect on cell proliferation seems particularly plausible, since the defect in hair cell patterning seen in the *Delta1* conditional knockout is strikingly similar to that seen in $p27^{Kip1}$ null mice. In both cases hair cells are produced prematurely and in excess, with an extra row of outer hair cells and extra inner hair cells. In the $p27^{Kip1}$ null cochlea the extra hair cells appear to be produced after increased proliferation of progenitor cells (Chen, Johnson et al. 2002). As discussed in the introduction, cells of the sensory patch switch on expression of $p27^{Kip1}$ between E12.5 and E13.5, a stage that matches the timing of their terminal mitosis. These cells form a zone of non-proliferating cells (ZNPC). This ZNPC can be visualised by staining sections of the cochlea with an anti-PCNA (proliferating cell nuclear antigen) antibody, which recognises a nuclear protein enriched in proliferating cells. In the $p27^{Kip1}$ knockout mouse, PCNA-positive cells are detected in the area of the

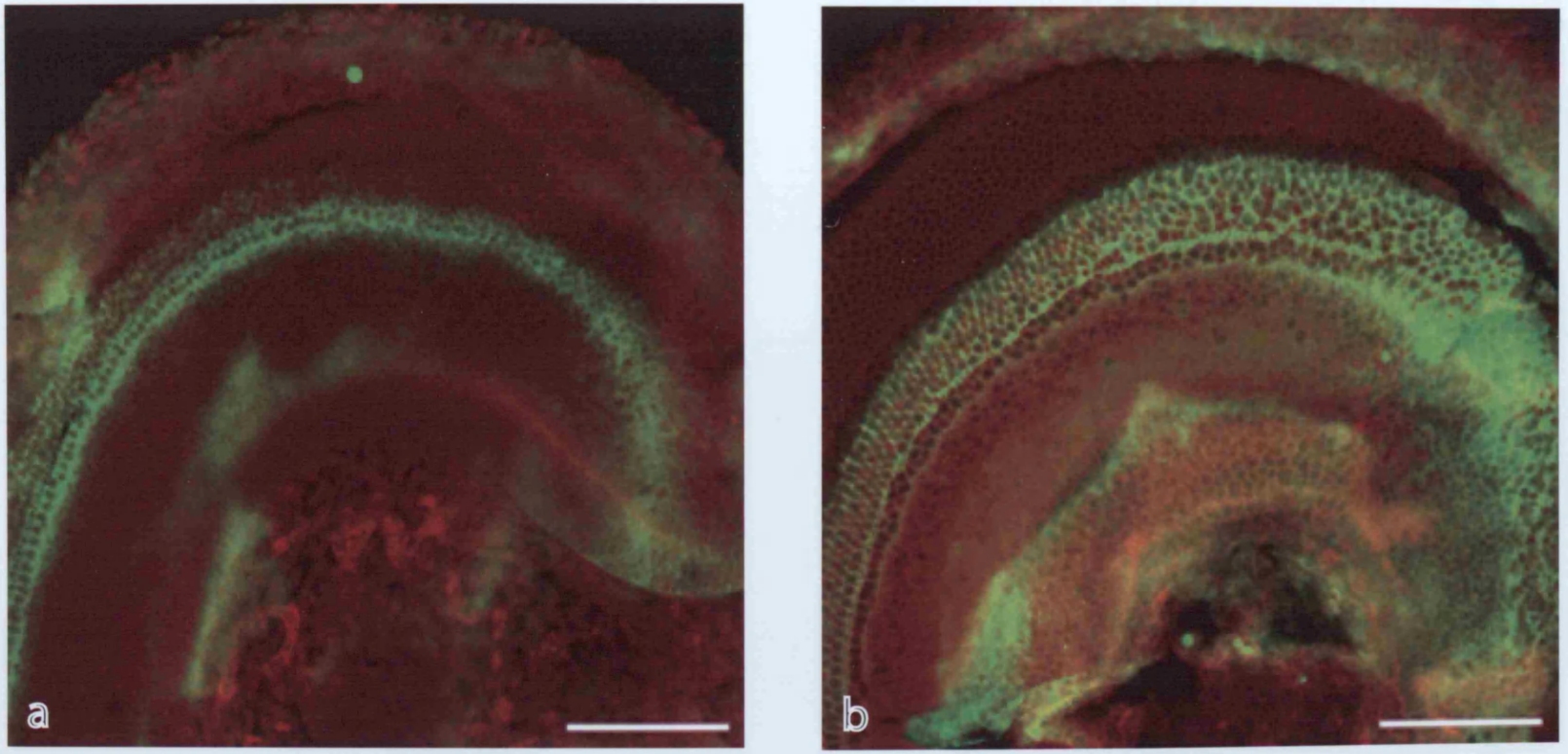


Figure 5.14

Supporting cells of the sensory patch are visualised using an antibody against Jagged1 (green) and a phalloidin counter stain (red) in the apex of the cochlea at E17.5 from a normal littermate (a) and a *Delta1* conditional knockout mouse (b). The sensory patch is clearly broader in apex of the mutant cochlea. Scale bars are 100µm.

Table 5.4

Hair cell and supporting cell counts in the apical part of the cochlea in *Delta1* conditional knockout mice at E17.5.

Litter numbers are inserted here only to identify littermates, and do not refer to other experiments.

Genotype	Litter No.	No. Hcs per. 100um	No. Scs per.100um	SC PER. HC
*Delta1*cko	Litter 1	74	188	2.5
*Delta1*cko	Litter 1	98	181	1.9
*Delta1*cko	Litter 2	81	141	1.7

sensory patch at E16.5, two days after all these cells would normally have exited the cell cycle, implying that cell division has continued abnormally (Chen, Johnson et al. 2002).

I investigated the possibility that a similar deregulation of proliferation in the sensory patch lay behind the hair cell phenotype in the *Delta1* conditional knockouts. To determine whether cells were still proliferating at this late stage in the *Delta1* conditional knockout, I collected litters at E17.5 and E15.5, and obtained cryosections of the head from conditional knockouts and their littermate controls. These sections were stained using the same anti-PCNA antibody as that used in the aforementioned analysis of the $p27^{Kip1}$ null mice (mouse monoclonal, clone P-10, Neomarkers).

The pattern of staining I saw in the cochlea of normal individuals was different to that previously described. The zone of non-proliferating cells appeared wider. PCNA staining was excluded from the area in which the sensory patch would form, as previously described, but was also absent from cells of the greater epithelial ridge medial to this. One reason for this could be that the conditions I used did not detect low levels of PCNA (Figure 5.15). With this proviso, my main finding was that there was no difference in PCNA staining between cochleas from control littermates and *Delta1* conditional knockout mice. In littermate controls at E15.5 and E17.5 the wide zone of non-proliferating cells could be seen at all apicobasal levels of the cochlea. This zone was also present, and was of similar width, in the *Delta1* conditional knockout cochlea. No PCNA positive cells were seen within this zone in any part of the cochlea at either of the stages examined. This, of course, does not exclude ectopic proliferation in the *Delta1* conditional knockout at earlier stages.

Due to the requirement for an antigen retrieval step before application of the anti-PCNA antibody, double staining with a marker of the sensory patch was not successful. I am therefore unable to rule out the possibility that there is a small amount of ectopic proliferation in the developing sensory patch. These results do, however, indicate that cell cycle control is not disrupted in the same way, or at least to the same extent, as observed in the $p27^{Kip1}$ mutant cochlea.

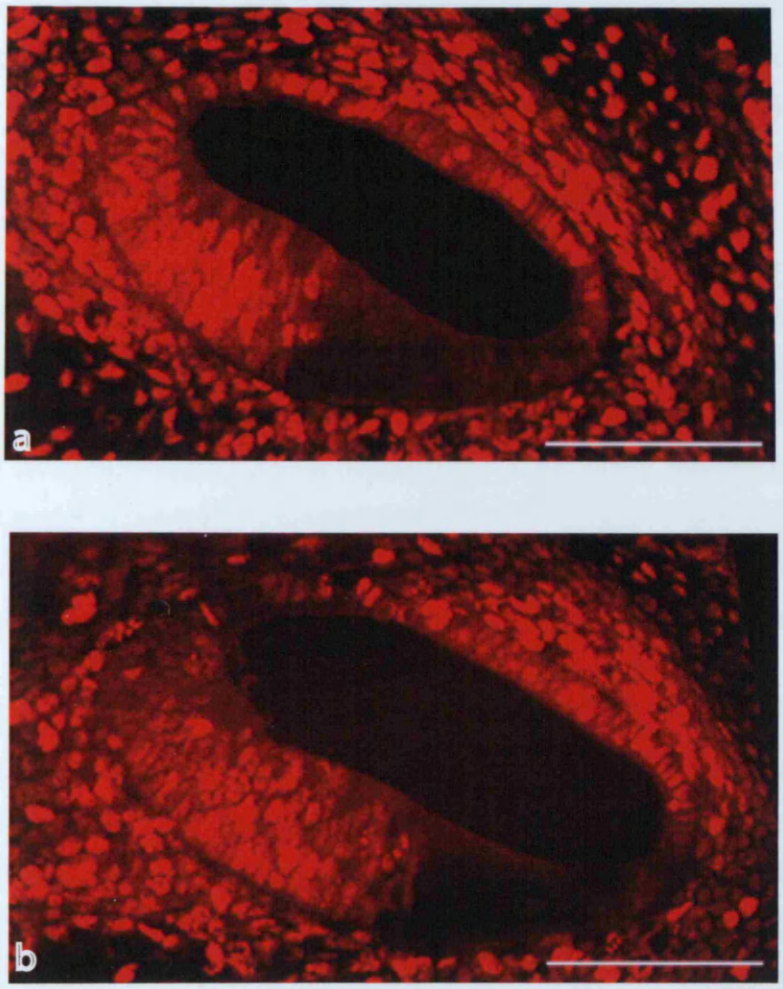


Figure 5.15

PCNA antibody stain in a section of the middle turn of the cochlea from an E15.5 *Foxg1-Cre* heterozygous littermate (a) and a *Delta1* conditional knockout mouse (b). Scale bars are 100μm.

5.2.9 *p27^{Kip1}* protein distribution is unaltered in the absence of *Delta1*

It remains possible that there has been a subtle abnormality in proliferation of sensory patch cells that cannot be detected in the above experiment. Only a small number of additional cell divisions would be required. I therefore wanted to further investigate the possibility that *Delta1* regulates proliferation in the sensory patch. Based upon the striking similarity of the hair cell phenotypes seen upon loss of *Delta1* and loss of p27^{Kip1} I thought that I might detect misregulation of p27^{Kip1} in the Delta1 conditional knockout, even though I had failed to see any effect by PCNA staining. I therefore looked at staining with the anti-p27^{Kip1} antibody in conditional knockout mice to check whether the levels of p27^{Kip1} protein were significantly altered after loss of *Delta1*.

I chose to study the pattern of p27^{Kip1} protein distribution in the wholemount cochlea at E14.5, 24 hours after the protein can first be detected (Chen, Johnson et al. 2002). I collected three litters containing *Delta1* conditional knockout mice at E14.5. I stained the cochleas from the conditional knockout mice and the appropriate littermate controls with the p27^{Kip1} antibody. All samples were stained in the same experiment, and were treated with the same solutions of primary and secondary antibodies, and the same series of washes. Cochleas were left whole for mounting and pictures were taken using the confocal microscope.

In the normal cochlea of control littermates at E14.5, the p27^{Kip1} antibody stains a broad domain at the very apex of the cochlea, and a narrower band of cells that extends along the length of the cochlea duct. This pattern of staining was also seen in all three *Delta1* conditional knockouts. Loss of *Delta1* did not appear to affect the distribution of p27^{Kip1} protein (Figure 5.16). The morphology of the *Delta1* conditional knockout cochlea differs from that of the control, as disussed previously, but the pattern of p27^{Kip1} remains the same. There were subtle differences in the levels of staining with the p27^{Kip1} antibody between individuals of the same genotype, despite efforts to keep conditions identical for each sample during the immunostaining process. I am therefore unable to rule out subtle changes in the levels of p27^{Kip1} in the *Delta1* conditional knockout. Thus if loss of *Delta1* does affect proliferation, it is likely to be by some mechanism acting independently of p27^{Kip1}. It remains possible that loss of Delta1 has some effect on cell proliferation.

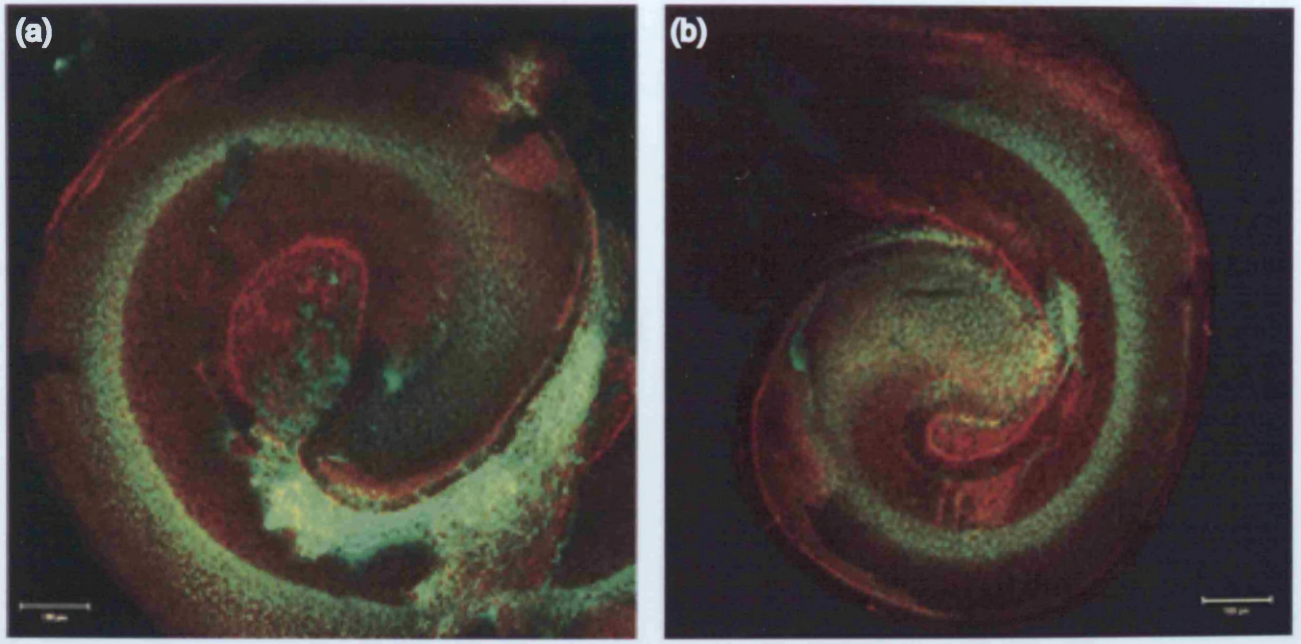


Figure 5.16

P27Kip1 antibody staining (green) in wholemount cochleas, counterstained with phalloidin (red). The pattern of p27Kip1 does not differ between control littermates (a) and *Delta1* conditional knockouts (b) at E14.5. Scale bars are 100μm.

5.3 Discussion

Analysis of the inner ear phenotype in the *Delta1* mutant has produced some results that support the proposed role of *Delta1* in the patterning of the sensory epithelium, but also some that appear to contradict it. I discuss the results concerning the vestibular patches first, then those concerning the sensory epithelium of the cochlea.

5.3.1 Loss of *Delta1* can result in loss of maculae

While the three cristae, and their associated semicircular canals and ampullae appear to be patterned normally, the saccular macula and/or the utricular macula are absent in the *Delta1* conditional knockout. Of the cases studied, both maculae were lost in one individual, while the saccular macula alone was lost in another two cases. The macula-specific effect may reflect a dual role for *Delta1* in the developing ear, specifying both neurons and hair cells. Neurons that will contribute to the cochleovestibular ganglion delaminate from the anteroventral part of the ototcyst between E9 and E10. *Delta1* would be expected to be lost throughout the otocyst from early stages in the conditional knockout mice, and is predicted to have been removed by the time neurogenesis occurs. Loss of *Delta1* in the developing ear may well result in excessive neurogenesis. Excessive production of neurons in the otocyst may then deplete a pool of precursor cells that later give rise the cells of the sensory patches. In support of this idea, the maculae seem to arise from the same area of the otocyst that neuroblasts delaminate from: both *Lfng* and *Jagged1* are expressed in the anteroventral part of the otocyst, where they are thought to mark the prospective maculae and the cochlea. The cristae derive from the posterior part of the otocyst, are marked by *Bmp4* (Morsli, Choo et al. 1998), and are unaffected in the *Delta1* conditional knockout,

Neurogenesis in the otocyst can be visualised using $Delta1^{LacZ}$ mice. Conditional knockout mice could be generated in which one copy of *Delta1* has already been replaced with the $Delta1^{LacZ}$ allele. Delaminating neuroblasts would then express *LacZ*. A simple X-Gal stain of the embryo at E10.5 to visualise this expression would then indicate whether excessive neuroblasts do indeed delaminate in the *Delta1* conditional knockout.

5.3.2 Early and excessive production of both hair cells and supporting cells occurs in the *Delta1* conditional knockout cochlea

Delta1 is expressed in developing hair cells in the inner ear, and is thought to deliver inhibitory Notch signalling to neighbouring cells, preventing them from developing as hair cells. Loss of *Delta1* would thus be expected to result in a loss of this inhibitory signal, allowing more cells to escape inhibition and develop as hair cells.

Some aspects of the patterning defect seen in the *Delta1* mutant mice fit with the proposed role of *Delta1*, others do not. In agreement with the model, hair cells are produced early and in excess when *Delta1* is lost. This abnormality in hair cell production is seen when Notch signalling is disrupted in zebrafish. In the zebrafish mutant, *mindbomb* (*mib*), loss of Notch signalling results in early and excessive production of hair cells at the expense of supporting cells (Haddon, Jiang et al. 1998). A dominant negative form of one of the zebrafish Delta genes, *deltaA*, causes a milder phenotype, where hair cell numbers are increased at the expense of supporting cells (Riley, Chiang et al. 1999). One explanation for the early production of hair cells in zebrafish is that the process of hair cell selection by lateral inhibition normally delays hair cell differentiation. When Notch signalling is lost, the selection process fails to occur, and cells are able to develop early as hair cells.

There are multiple Notch ligands expressed in developing hair cells in the zebrafish ear. These ligands appear to act in a redundant fashion, so that disruption of one does not completely phenocopy the *mindbomb* mutant. A similar situation exists in mouse, where *Delta1* and *Jagged2* are both expressed in developing hair cells in the inner ear (Lanford, Lan et al. 1999; Morrison, Hodgetts et al. 1999). A partial effect may be seen upon loss of one of these ligands expressed in developing hair cells. Thus, it is not surprising that in the *Delta1* conditional knockout there is a mild overproduction of hair cells.

However, the mouse mutant differs from the zebrafish in that the excess hair cells do not appear to be produced at the expense of supporting cells. This is a surprising result, which demands a reassessment of the role of *Delta1* in hair cell patterning, and perhaps of how the sensory patch develops in the mammalian cochlea. First, however, I will examine more carefully some of the findings on which my conclusions are based.

5.3.3 There are limitations to the cell counting approach used in this study

The cell counts I performed in this study were limited to the supporting cells associated with the outer hair cells, and excluded those associated with the inner hair cells. This was necessary as staining with the Jagged1 antibody did not provide a clear demarcation between supporting cells in this area and adjacent cells in the greater epithelial ridge. I cannot, therefore, make definite statements about the effect of loss of *Delta1* on the total numbers of hair cells and supporting cells in the sensory patch. I can only do so in the outer hair cell region.

Another qualification is that the length of the cochlea is reduced in the *Delta1* conditional knockout, and the coiling of the cochlea is delayed. Thus, it is conceivable, though perhaps far fetched, that the total number of hair cells is not increased, but that their spatial arrangement has been altered. Counting the total number of hair cells in the cochlea of control and *Delta1* conditional knockout mice would not resolve this issue, as the overall size of the *Delta1* mutant mice is reduced.

5.3.4 A defect in outgrowth of the cochlea may contribute towards the hair cell patterning defect

The pattern of hair cells in the cochlea of *Delta1* conditional knockout mice at E17.5 resembles that seen in the *loop-tail* (*Lp*) mutant mouse cochlea. *Lp* is a vertebrate homologue of the Drosophila gene *Van Gogh*, which is involved in determining planar cell polarity (PCP), and in convergent extension during development. Homozygous *Lp* mutant mice exhibit a strong open neural tube defect, and die around birth (Montcouquiol, Rachel et al. 2003). The cochlea from these mice is reduced in length, being 27% shorter than those of wildtype littermates, suggesting that convergent extension of the cochea is disrupted. In addition to the expected defects in hair cell planar polarity, an excess of hair cells is seen in the apex of the cochlea, with an incomplete extra row of inner hair cells, and 6 clearly identifiable rows of outer hair cells. These findings raise the possibility that the defect I observe in outgrowth of the cochlea contributes to the broadening of the sensory patch at the apex of the cochlea of *Delta1* conditional knockout mice. I am not aware of any evidence, however, that Delta1 acts in the planar cell polarity pathway, or in convergent extension.

5.3.5 There are several possible mechanisms for expansion of the sensory patch

There are a number of cases in the literature where the sensory patch is expanded, or where phenomena are reported that suggest how this might occur. With these cases in mind, I describe several different mechanisms that might give rise to the increase in the size of the sensory patch in the cochlea upon loss of *Delta1*.

Recruitment of extra hair cells and supporting cells

The sensory patch may be enlarged by recruitment of additional hair cells and supporting cells at the edge of the patch. A recruitment of non-sensory cells to the patch has been observed in cultures of late embryonic and early postnatal cochleas taken from wildtype rats, where an extra row of hair cells and supporting cells was formed spontaneously at the outer margin of the patch, apparently without an increase in proliferation (Lefebvre, Malgrange et al. 2001; Malgrange, Thiry et al. 2002). Extra rows of outer hair cells and of Deiters' cells were produced at the expense of epithelial cells adjacent to the organ of Corti. Ectopic expression of *Math1* and *Jagged2* was detected in the region where supernumary hair cells were produced, suggesting that these cells had the molecular characteristics of normal hair cells. The numbers of the extra hair cells and supporting cells increased upon treatment of the cultured cochleas with growth factors (Malgrange, Thiry et al. 2002). Such transformations of cell fate are also produced by application of chemical inhibitors of CDKs (Malgrange, Thiry et al. 2002; Malgrange, Knockaert et al. 2003).

While this mechanism of expansion of the sensory patch in this manner cannot be ruled out in the *Delta1* mutant, it does not fit with previous work on *Delta1*. Also, it does not explain the premature differentiation of hair cells seen in the *Delta1* conditional knockout.

Production of extra hair cells and supporting cells by increased proliferation of progenitors

An increase in hair cell production, strikingly similar to that seen in the *Delta1* conditional knockout, is seen when inhibition of proliferation is removed by loss of $p27^{Kip1}$. Both *Delta1* and $p27^{Kip1}$ knockout mutants exhibit an extra row of outer hair cells and increased numbers of inner hair cells in the organ of Corti (Chen and Segil 1999; Lowenheim, Furness et al. 1999). The extra hair cells seen in the $p27^{Kip1}$ mutant seem to arise as a consequence of increased proliferation of progenitor cells. Cells within the sensory patch can be seen to be

proliferating in the *p27^{Kip1}* mutant several days after cells of the sensory patch have normally exited the cell cycle (Lowenheim, Furness et al. 1999). This suggests that *p27^{Kip1}* in the sensory patch is required to prevent progenitor cells from re-entering the cell cycle. The surplus cells produced in the absence of *p27^{Kip1}* may interact with one another in the normal fashion to give a mix of hair cells and supporting cells, both in excess but in a roughly normal ratio.

I investigated the possibility that *p27^{Kip1}* might be a downstream target of *Delta1* by looking at the antibody staining pattern for this protein in the *Delta1* conditional knockout cochlea. The staining pattern with an anti-*p27^{Kip1}* antibody was not altered in conditional knockout mice compared to littermate controls at E14.5. This indicates that *Delta1* is not required for transcriptional regulation of *p27^{Kip1}* in the developing sensory patch, and suggests that it is not required for maintaining normal levels of p27^{Kip1} protein. This does not, however, rule out the possibility that Delta1 serves at some other stage to switch on *p27^{Kip1}*. Because I have only looked at one time point, which is a day after cell proliferation would normally cease (Ruben, 1967), I cannot rule out the possibility that *p27^{Kip1}* activity is delayed, or is at lower levels initially in the absence of *Delta1*. Also, it may be that Notch activation by *Delta1* results in post-transcriptional modification *p27^{Kip1}*.

While expansion of the sensory patch in the *Delta1* mutant by increased proliferation cannot be ruled out, it does not fit with previous work on *Delta1*, or with my own data.

An excess of hair cells due to a loss of lateral inhibition may stimulate production of additional supporting cells

It is not obvious how the above possibilities – that is, recruitment of cells from the adjacent non-sensory epithelium, and abnormal proliferation of prosensory cells- could relate to the known function of Delta1. There is, however, a simple explanation of the phenotype I saw that would fit with the established role of Delta1 in lateral inhibition: loss of *Delta1* reduces the amount of lateral inhibition delivered by developing hair cells, causing hair cells to be produced in excess (at the expense of supporting cells) and prematurely, as observed.

What could then explain the accompanying increase in the number of supporting cells observed in the *Delta1* conditional knockout cochlea?

Extra supporting cells might be recruited by the extra hair cells. Hair cells have been shown to be capable of inducing neighbouring cells to form supporting cells (Woods, Montcouquiol et al. 2004). Solitary ectopic hair cells, produced by misexpression of *Math1* in the epithelial cells outside the sensory patch, cause their neighbouring cells to become supporting cells, as judged by their expression of the supporting cell markers *Jagged1* and *otogelin*. In this way the balance of hair cells and supporting cells would be re-established after excessive hair cell production.

Alternatively, extra hair cells might stimulate the depleted population of supporting cells to proliferate, and thus rebalance the ratio of hair cells and supporting cells. This idea is supported by the finding that supporting cells are able to re-enter the cell cycle during regeneration of hair cells in the chick inner ear. Such behaviour of supporting cells is not observed in the mature mammalian cochlea. However, there is a window of opportunity in the late embryonic and early postnatal cochlea when mammalian hair cells can be replaced. In the embryonic cochlea, replacement of the hair cells after laser ablation occurs without proliferation of cells of the sensory patch (Kelley, Talreja et al. 1995). However, hair cell regeneration in the neonatal cochlea was occasionally achieved after neighbouring cells re-entered the cell cycle.

This interpretation of the *Delta1* conditional knockout phenotype – that hair cells are produced in excess, and that residual supporting cells proliferate to rebalance the ratio of the different cell types – is further supported by observations of A. Kiernan et al, reported at a recent conference (Molecular Biology of Hearing and Deafness, Baltimore, October 2004). They have produced double mutant mice, which are homozygous for both a knockout mutation of *Jagged2*, and a hypomorphic allele of *Delta1*. They saw a phenotype similar to my *Delta1* conditional knockout mice, though with a greater excess of hair cells than that seen in either *Jagged2* or *Delta1* single knockout cochleas. Careful analysis of proliferation in the organ of Corti revealed ectopic proliferation in the sensory patch of these mutant mice.

The above suggestions are both plausible, and it is possible that the expansion of the sensory patch seen upon disruption of Notch signalling occurs by more than one of the mechanisms described. The most likely mechanism behind the expansion of the patch in the *Delta1* conditional knockout seems to be that hair cells are produced in excess upon loss of lateral inhibition, at the expense of the supporting cells, and that extra supporting

cells are subsequently produced through an expansion of the population of supporting cells within it. Future experiments ought to include careful analysis of proliferation in the developing sensory patch to determine whether there is abnormal proliferation in the absence of *Delta1*.

5.3.6 Why are extra supporting cells generated in mouse but not in fish?

A key question is why extra supporting cells are produced to accompany the extra hair cells produced upon disruption of Notch signalling in mice, but not in zebrafish.

In the zebrafish mutant *mindbomb* (*mib*), a failure of lateral inhibition results in an early and excessive production of hair cells. The supporting cell population appears to be entirely lost, and hair cells are extruded from the epithelium in their absence (Haddon, Jiang et al. 1998; Haddon, Mowbray et al. 1999). In this case, the loss of the entire supporting cell population is caused by a complete failure of lateral inhibition. No residual supporting cells would then remain to repopulate the sensory epithelium.

The ear phenotype of zebrafish carrying a dominant negative allele of *deltaA*, *deltaA*^{dx2}, is more difficult to reconcile with the interpretation of the *Delta1* conditional knockout phenotype (Riley, Chiang et al. 1999). Homozygous *deltaA*^{dx2} fish display an increase in hair cell numbers, accompanied by a decrease in supporting cell numbers.

5.3.7 How does the phenotype of the *Delta1* conditional knockout mice fit with studies of the role of *Delta1* in chick?

The theory that I have put forward as most plausible for my *Delta1* data would also fit with observations in chick, where *Delta1* was misexpressed in the developing patches. No change in the pattern of hair cells and supporting cells was seen in patches of cells overexpressing *Delta1* (Eddison, Le Roux et al. 2000). However, a subtle increase in the size of the sensory patches in which *Delta1* was misexpressed would not have been detected in these experiments. It may be that extra hair cells were produced in these patches, but that secondary proliferation or recruitment of supporting cells re-established the alternating pattern of the different cell types.

5.3.8 The *Delta1* knockout phenotype differs from that of *Jagged2* knockout mice

A striking feature of the *Delta1* conditional knockout phenotype in the cochlea is the premature production of hair cells, at least in the apex of the cochlea. This is clearly seen in the wholemount cochlea of *Delta1* mutants at E17.5. Lanford et al (1999) collected *Jagged2* knockout mice at a similar time point, at E18, but did not report an early production of hair cells, though the slightly later time of collection may have obscured this aspect of the phenotype (Lanford, Lan et al. 1999).

Moreover, the *Delta1* and *Jagged2* knockout phenotypes clearly differed in the numbers of extra outer hair cells produced. While there was a complete extra row of outer hair cells throughout the middle and basal portions of the *Delta1* conditional knockout cochlea, and multiple extra rows in the apical portion, an extra row of outer hair cells was seen only occasionally in the *Jagged2* knockout cochlea. The increase in hair cell numbers seen upon loss of *Jagged2* was largely due to an excessive production of inner hair cells (Lanford, Lan et al. 1999).

The more severe overproduction of hair cells seen in the apex of *Delta1* conditional knockout mice was not reported in *Jagged2* knockouts. While Lanford et al did not analyse the production of hair cells in wholemount *Jagged2* knockouts, they did analyse sections of the apex of the cochlea from E17.5 individuals (Lanford, Shailim et al. 2000). A mild overproduction of hair cells, similar to that seen in more basal regions of the cochlea, was seen. Their analysis on sections showed morphologically identifiable hair cells in the apex of control cochleas at this stage, whereas I did not find this to be the case in my experiments. This may reflect differences in the developmental timing between different strains of mice, or perhaps indicates that they did not examine hair cell production in the extreme apex of the cochlea.

It is possible that the difference in the two phenotypes might be due to the use of *Foxg1-Cre* mice to recombine *Delta1*^{flox}. Loss of *Delta1* in the conditional knockout mice occurs on a *Foxg1* heterozygous background, and it is possible that loss of one copy of the *Foxg1* gene contributes to the phenotype observed. However, I saw no defect in hair cell production in the *Foxg1* heterozygotes I used as control littermates in my experiments.

In conclusion, the overproduction of hair cells seen in the *Jagged2* mutant mice was not as severe as that seen upon loss of *Delta1*. There is no evidence that hair cells were produced early in the *Jagged2* knockout, though the available data does not exclude the possibility. Further analysis of the timing of hair cell production in the *Jagged2* knockout cochlea is required to determine whether the two ligands perform different roles in hair cell patterning.

The possibility remains that the differences between the *Jagged2* and *Delta1* knockout phenotypes reflect a qualitative difference in their roles in patterning the sensory epithelium, although in both cases it seems likely that the phenotype is due at least partly to a reduction in lateral inhibition when nascent hair cells express Notch ligands at a reduced level.

5.3.9 Why have two ligands in hair cells?

It may be that Delta1 and Jagged2 activate Notch in the same way, but act at different stages of hair cell development. Different functions may thus stem from a difference in the temporal regulation of these two ligands. *Delta1* is expressed transiently, whereas *Jagged2* is expressed more persistently in hair cells of the mouse cochlea, becoming progressively downregulated only after P3 (Lanford, Lan et al. 1999). In chick, this difference in expression pattern appears to be more pronounced, with *Jagged2* being expressed and maintained in mature hair cells (unpublished data, this lab).

Perhaps the two ligands perform overlapping roles in lateral inhibition, with *Delta1* being the more important ligand involved at early stages leading up to hair cell commitment, and *Jagged2* playing a later role in maintaining the pattern of hair cells and supporting cells.

Chapter 6

Discussion

6.1 *Jagged1* and *Delta1* play complementary roles in the development of the inner ear

A striking feature of the different phenotypes in the *Jagged1* and *Delta1* conditional knockout mice is the almost opposite effects upon patterning of the sensory patches. In the vestibular patches, loss of *Jagged1* affects the cristae most severely, while loss of *Delta1* affects the maculae most severely. Also, the two types of mutation have seemingly opposite effects on the production of hair cells in the cochlea. Loss of *Jagged1* results in a dramatically reduced sensory patch, whereas loss of *Delta1* results in an enlarged patch.

An explanation of these contrasting phenotypes can be given based upon three premises: (1) both Delta1 and Jagged1 activate Notch; (2) activation of Notch in a given cell inhibits expression and/or activity of Delta1, but stimulates expression of *Jagged1*; and (3) Notch has two functions in the inner ear: it acts initially to drive cells to adopt a prosensory character, and subsequently inhibits them from differentiating as hair cells.

The *Jagged1* knockout ear phenotype can then be explained as follows. Jagged1 acts early to promote prosensory development by activating Notch, so that its loss leads to reduction of the prosensory domain. This is manifest in an extreme way in some vestibular patches (two of the cristae are completely lost), and in a milder way in the cochlea (the outer but not inner hair cells are lost). At a later stage in sensory patch development, Jagged1 may have a second role in limiting hair cell production.

The *Delta1* knockout phenotype, on the other hand, can be interpreted entirely in terms of the lateral-inhibition effects on cell fate determination within the prosensory patches. The loss of some vestibular patches may result from failure of lateral inhibition during neurogenesis, depleting the pool of prosensory cells. Later, when hair cells are being specified, a reduction of lateral inhibition upon loss of *Delta1* results in premature and excessive production of hair cells. This function of Delta1 is partially redundant with that of

Jagged2. Additional supporting cells, I suggest, are subsequently produced as a secondary effect of the excess of hair cells.

6.2 Future Work

I have addressed the future work to be conducted on the function of *Jagged1* and *Delta1* in ear development in their respective chapters. Here I discuss some more general questions about Notch signalling in ear development.

6.2.1 How are hair cells specified?

My data support the theory that Notch ligands are required to pattern hair cells through a process of lateral inhibition. Lateral inhibition at its simplest, based solely upon a negative feedback loop between the Notch receptor and its ligand, could establish the alternating pattern of hair cells and supporting cells. However, as discussed in the introductory chapter of this thesis, there are several factors that may bias the outcome of competitive Notch signalling, by affecting a cell's ability either to receive or to deliver Notch activation. Some of these factors are known to play a role in cell fate determination in other systems, though their function in the ear – if any - is not yet known.

An example of a factor that affects a cells ability to receive Notch activation is Numb. As described in the introduction, experiments in Drosophila have shown that Numb is a negative regulator of Notch signalling, and is asymmetrically inherited so as to bias the outcome of later Notch-mediated cell fate decisions (Uemura, Shepherd et al. 1989; Rhyu, Jan et al. 1994). It is expressed in the developing sensory patch in chick, and is asymmetrically distributed between the different cell types, with higher levels of Numb protein detected in hair cells (Eddison, Le Roux et al. 2000). Asymmetrical distribution of Numb might therefore effectively predetermine which cells will escape inhibitory Notch signalling and develop as hair cells by blocking reception of a Notch signal.

A cell's ability to deliver Notch activation may also be biased by *mindbomb/neuralised.* Both factors promote Notch signalling by acting upon Delta protein in the cell delivering activation Delta (Parks, Kleug et al. 2000; Deblandre, Lai et al. 2001; Lai, Deblandre et al. 2001; Yeh, Demer et al. 1997; Pavlopoulos, Pitsouli et al. 2001; Seugnet, Simpson et al. 1997), and

asymmetrical distribution of Neuralised has been shown to govern some cell fate decisions in Drosophila sensory development (Le Borgne and Schweisguth 2003). As described previously, loss of *mindbomb* results in a failure of lateral inhibition in the zebrafish ear (Haddon, Jiang et al. 1998). Asymmetrical distribution of such factors would thus increase the ability of a subset of cells to deliver Notch activation, allowing them to inhibit the hair cell fate in their neighbours, and to develop as hair cells themselves.

6.2.2 Are other Notch receptors involved in sensory patch development?

An increase in the number of hair cells in the mouse cochlea is seen upon loss of *Notch1*, *Delta1* or *Jagged2*. This suggests that Delta1 and Jagged2 in developing hair cells deliver lateral inhibition to neighbouring cells by interaction with Notch1. As Notch1 appears to be act only in lateral inhibition, the receptor through which Jagged1 acts to promote prosensory fate early in development remains to be identified.

An analysis of the expression of various Notch pathway members in the developing mouse inner ear concluded that *Notch2*, *Notch3* and *Notch4* are not expressed in the developing sensory epithelia (Lewis, Frantz et al.1998). However, this paper also asserted that *Delta1* was not expressed in the developing patches, though later experiments showed that *Delta1* is present, but is difficult to detect (Morrison, Hodgetts et al. 1999). A more thorough analysis of the expression of the Notch genes is required to complete the picture of how Notch signalling patterns this tissue.

My own preliminary experiments indicate that at least one other Notch receptor is expressed in the sensory patch: *Notch3*. In situ hybridisation on sections of the mouse inner ear at E15.5 detects expression of *Notch3* in the supporting cells of the saccular macular (Figure 6.1). This is a preliminary result, and further analysis is required to determine whether *Notch3* is expressed early in the otocyst, and how its expression relates to that of *Jagged1*.

It is quite possible that Jagged1 interacts with Notch3 in inner ear development. An assay of the binding affinity of Jagged1 for Notch1-3 indicated that Jagged1 bound to each receptor, but with differing affinity. The strongest interaction was with Notch3, then Notch2, and then Notch1 (Shimizu, Chiba et al. 1999). However, the effects of such an interaction

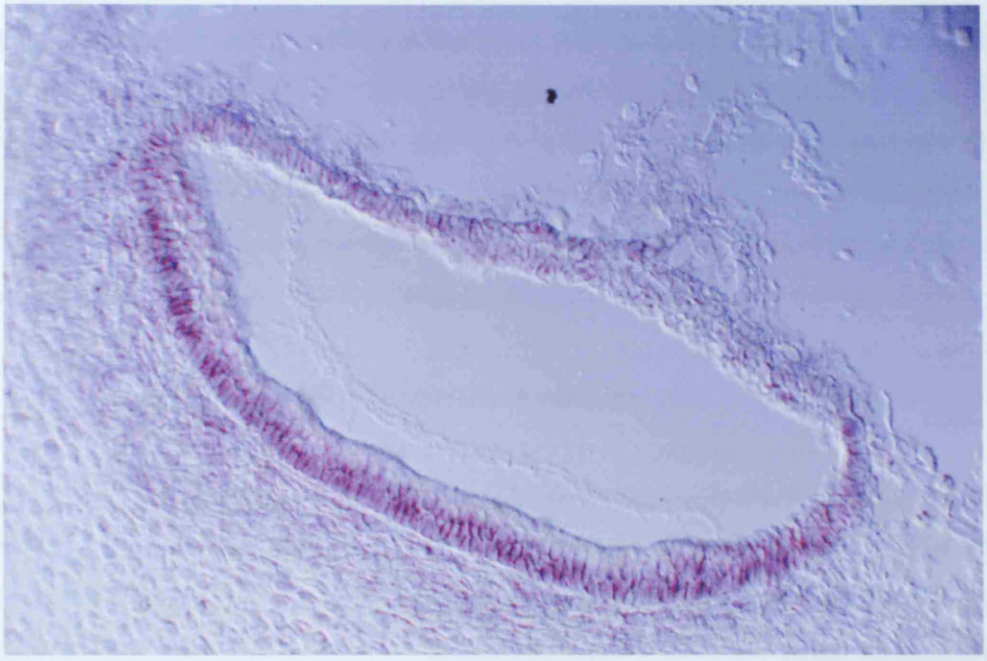


Figure 6.1
Notch3 in situ hybridisation on a section of the wildtype mouse saccular macula at E15.5.

are difficult to predict, as there is at least one report that Notch3 antagonises the activity of other Notch proteins.

6.2.3 Which direct targets of Notch signalling mediate its early function in sensory patch specification?

The downstream targets of Notch signalling include the Hes and Hey families of bHLH transcription factors. Two Hes family members, Hes1 and Hes5, have been implicated as targets of inhibitory Notch signalling in the inner ear. Both *Hes1* and *Hes5* knockout mice exhibit an excess of hair cells in the cochlea, specifically affecting the inner and outer hair cells respectively (Zine, Aubert et al, 2001). This finding suggests that Hes1 and Hes5 are downstream targets of Notch1, activated by Delta1/Jagged2. However, there is as yet no evidence as to which are the most likely candidates among the bHLH transcription factors for mediating the early, prosensory function of Notch.

6.2.4 What are the other factors involved in sensory patch specification?

The different sensory patches in the inner ear vary in their requirement for Jagged1 in prosensory patch specification; some can develop normally even in its absence. Which factors drive sensory patch development in the absence of *Jagged1*? Many transcription factors and signalling molecules have been implicated in sensory patch specification. A loss or reduction of sensory patches is seen in mice with mutations in Pax genes, Six genes, Eya genes, Dlx genes, Bmps and Fgfs, as discussed in the introductory chapter of this thesis. It is conceivable that these factors interact with the Notch signalling pathway in determining prosensory patches of the inner ear. Further analysis of the respective functions of these other factors in sensory patch development and the nature of their interaction with the Notch pathway in sensory patch specification is required.

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